





THE JOURNAL  
OF  
PHARMACOLOGY  
AND  
EXPERIMENTAL THERAPEUTICS

FOUNDED BY JOHN J. ABEL

OFFICIAL PUBLICATION  
OF THE AMERICAN SOCIETY FOR PHARMACOLOGY AND  
EXPERIMENTAL THERAPEUTICS INCORPORATED

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VOLUME 73

1941

BALTIMORE, MARYLAND

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JOHNSON REPRINT CORPORATION  
111 Fifth Avenue, New York 3, New York

Johnson Reprint Company Limited  
Berkeley Square House, London, W. 1.



First reprinting, 1963, Johnson Reprint Corporation



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# THE SWEAT RESPONSE TO DRUGS WITH NICOTINE-LIKE ACTION<sup>1,2</sup>

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Received for publication April 15, 1941

In a previous communication (1) the local pilomotor response to intradermal injection of acetylcholine was described. The conclusion was drawn that this reaction is due to the nicotine-like action of acetylcholine and is effected through an axon reflex mechanism within the skin. Reference was also made to a localized sweat response accompanying the pilomotor activity. The purpose of this paper is to describe further experiments carried out to determine the mechanism of this sweat response.

## EXPERIMENTAL

### *Sweat responses of human skin to drugs with nicotine-like action*

In these experiments Minor's iodine-starch method (2) was used to visualize the sweat secretion. All drugs studied were brought to the specified dilution with 0.9 per cent NaCl. Acetylcholine was used as the bromide, nicotine as the sulfate, and alpha-lobeline as the hydrochloride. All intradermal injections were made with volumes of 0.1 to 0.2 cc. All subjects were normal white males between the ages of 20 and 35. Basic observations were made upon 50 students, and more detailed experiments were carried out and repeated on the authors as subjects.

Intradermal injection of acetylcholine (3) or mecholyl (4) in the human elicits on the wheal a sweat secretion which is due to the direct muscarine-like action of acetylcholine on the sweat glands. This response appears within 5 to 10 seconds and slowly increases in intensity and in extent corresponding to the slow diffusion of the drug. The maximum spread, even with high concentrations (1:1000 to 1:10,000), is only a few millimeters away from the wheal and is not reached until 5 to 10 minutes after injection. The intensity of this reaction decreases with decreasing concentration of the drug.

<sup>1</sup> Cf. preliminary report in the Proceedings of the Society for Experimental Biology and Medicine, 1939, 42: 231.

<sup>2</sup> This work was aided in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago, and by the Commonwealth Fund.



We noted that this response can be elicited even by concentrations as low as 1:50,000,000. In addition, we found that concentrations of 1:10,000 to 1:80,000 of acetylcholine produced still another type of sweat response, distinct and separate from the one described above. This was a sudden widespread outbreak of sweat droplets around the injection wheal in an area about 5 cm. in diameter, with occasional isolated groups of secreting pores outside the main responding area. This response appeared immediately, was fully developed within 1 to 2 minutes after injection, and could easily be distinguished from the slowly spreading sweating caused by the direct muscarine-like action.

A comparison of this sweat response with the "goose flesh" occurring at the same time showed that the areas involved were approximately the same in both extent and shape. However, the responding areas did not coincide completely around their irregularly shaped edges where isolated groups of hair follicles and sweat pores were seen in action separately. The "goose flesh" appeared in most cases during the injection, whereas the sweat response began to appear about 5 seconds later. The pilomotor reaction subsided within 2 minutes. Similarly, the sweat glands ceased to function in about 2 minutes, as was shown by applying the indicator at different intervals after the injection to determine the minimum time after which no black spots appeared.

The reaction was strongest in a warm environment close to the threshold of sweat gland activity. Only slight responses were obtained when the subject was well chilled in a refrigerator room at a temperature of  $-3^{\circ}\text{C}$ . Individual differences corresponded to variation in tendency to perspire physiologically.

The similarity of the sweat and pilomotor responses suggested that they are controlled by similar mechanisms. Accordingly, since the pilomotor reaction to acetylcholine has been proved to be due to its nicotine-like action, the sudomotor effects of intradermal injections of nicotine sulfate and alpha-lobeline hydrochloride were tested on human skin prepared with iodine and starch. Nicotine sulfate (1:100,000) and alpha-lobeline hydrochloride (1:1,000,000) produced sweat responses identical with the widespread response to acetylcholine (fig. 1). Thus the dual nature of the sudomotor effect of intradermal acetylcholine became apparent. The nicotine-like action of acetylcholine in concentrations of 1:10,000 to 1:80,000 caused a sudden widespread sweat response identical with the response to nicotine, whereas the muscarine-like action elicited a slowly developing response only at the immediate site of the injection (fig. 2, a, b).

The nicotine and muscarine sudomotor effects could be further differentiated by the different way in which they were influenced by local anesthetics or by large doses of drugs with nicotine-like action. If an effective concentration





FIG. 1. SWEAT RESPONSE TO INTRADERMAL INJECTION OF 1:100,000 NICOTINE-SO<sub>4</sub>. VISUALIZED BY MINOR'S METHOD

(a)  
Acetylcholine-Br  
1:40,000



(b)  
Nicotine-SO<sub>4</sub>  
1:100,000



Nicotine-SO<sub>4</sub> 1:100,000  
+  
Procaine-HCl 1:100,000



(c) Acetylcholine 1:40,000  
+  
Procaine-HCl 1:100,000

1. Nicotine-SO<sub>4</sub> 1:1000  
2. Nicotine-SO<sub>4</sub> 1:100,000



(e)

FIG. 2. DIAGRAMMATIC REPRESENTATION OF THE SWEAT RESPONSES TO ACETYLCHOLINE AND NICOTINE (a and b), AND THE EFFECT THEREON OF MIXING THESE DRUGS WITH PROCAINE (c and d) OR OF PREVIOUS INJECTION OF HIGH CONCENTRATION OF NICOTINE (e). SEE TEXT



of acetylcholine was injected into an area of skin anesthetized by subcutaneous infiltration of procaine hydrochloride, the usual sudden outbreak of sweat droplets several centimeters around the wheal was abolished but they still appeared directly on the wheal due to the direct muscarinic action on the glands (fig. 2, d). The sweat response to nicotine was totally abolished not only by previous infiltration of the skin with procaine but also by mixing the nicotine solution before injection with procaine solution as dilute as 1:100,000 (fig. 2, c).

If concentrations of nicotine greater than 1:1000 or of alpha-lobeline greater than 1:100,000 were injected intradermally not only did these fail to produce a sweat response but subsequent injections at the same point of otherwise effective concentrations of these two drugs also caused no effect (fig. 2, e). However, acetylcholine injected at the same point still caused a sweating on the wheal because of the direct action of its muscarinic component on the gland cells.

Pilocarpine hydrochloride, when injected intradermally in any concentration down to 1:400,000, elicited a sweating only on the wheal and in the immediate vicinity. This reaction was not abolished by local anesthetics or by high concentrations of nicotine. There was no evidence of any effect similar to that produced by drugs with a nicotine-like action. This result confirmed the still challenged view (5) that pilocarpine acts directly on sweat gland cells.

#### *Sweat responses in the cat's paw*

Adult cats with soft foot pads were found to be the best subjects for these experiments. Drug volumes of 0.2 cc. were injected subcutaneously into the toe pad. In this work 24 animals were used. Sweat responses of the cat's paw to local injections depended largely on the state of the horny layer in the skin of the pads. In hyperkeratotic toe pads responses, even to high concentrations of pilocarpine or acetylcholine, were questionable or very inconsistent.

According to Langley (6) and Langley and Uyeno (7) injection of Ringer's solution into a pad of a cat's paw causes a slight fleeting sweat secretion on the pad. However, these authors found that this response could easily be distinguished from the more profuse and more lasting reaction to pilocarpine. Similarly, it was found in our experiments that the outbreak of sweat on the foot pads was greater after injection of nicotine or alpha-lobeline than after the control injections of physiological saline solution. Most adult cats with soft foot pads responded slightly to injection of saline controls and quite strongly to that of 1:200,000 nicotine or of 1:1,000,000 alpha-lobeline. In many cats the saline control had no effect, while nicotine and alpha-lobeline produced a definite response. The response to saline was always limited to a small part of the area of the pad, while responses to drugs with



nicotine-like action generally involved the whole surface of the injected toe pad.

The results with the cat were similar to those in man. Optimal responses were obtained with 1:200,000 nicotine and with 1:1,000,000 alpha-lobeline. The sweat drops were seen with a magnifying glass to appear 5 to 10 seconds after the injection and could be observed to disappear by evaporation in about 1 minute. The intensity and duration of this reaction depended on the room temperature and on the condition of the foot pads. The response was abolished by previous infiltration of the pad with local anesthetics or with 1:1,000 nicotine. It failed to appear if effective doses of nicotine were mixed with local anesthetics before injection.

#### *Mechanism of the sweat response to drugs with nicotine-like action*

The similarity between the pilomotor and the sweat reactions with respect to their time of onset, rate and extent of spread, and duration suggested that the sweat reaction described above is mediated through an axon reflex mechanism, as has already been demonstrated for the pilomotor reaction. Evidence for the axon reflex nature of the sweat reaction was obtained by experiments (a) with local anesthetics, (b) by fresh sectioning of the nerves, and (c) by the use of freshly excised skin.

(a) Local anesthetics, as we have seen, abolished the sudomotor effect of nicotine when the skin area to be tested was anesthetized by direct infiltration. However, block anesthesia produced by procaine injection around the lateral antibrachial cutaneous nerve in man did not abolish the response in the resulting anesthetized area. (b) Similarly, immediately after sectioning of the sciatic nerve or extirpation of the lumbar and sacral sympathetic chain in the cat, there was no impairment of the sweat response in the hind paws. (c) The toe pads of anesthetized cats were excised and injected immediately with 1:200,000 nicotine. An outbreak of sweat droplets occurred if the injection was made within three minutes after excision. After this interval there was no longer any reaction to nicotine but pilocarpine, which acts directly on the sweat glands, was effective after 10 minutes.

A further criterion of local nervous impulses is their disappearance after nerve degeneration. Concerning the sweat response to nicotine such evidence has been incomplete up to the present time.

By courtesy of Drs. D. B. Phemister and K. S. Grimson tests were made on five patients who for therapeutic purposes had undergone postganglionic sympathectomy in segments supplying the limbs. These tests were performed 4 weeks to 3 months after operation. In none of these cases did the denervated areas respond by sweating either to heat or to nicotine. However, this was not considered good evidence for the nervous nature of the response because the same areas did not respond to the supposedly direct action of



pilocarpine (1:1,000) or of acetylcholine (1:1,000). A failure of the sweat response to pilocarpine and mecholyl in sympathectomized skin areas has often been reported (5) whereas similar observations concerning acetylcholine could not be found in the literature. These findings are remarkable since strong evidence has been presented for the direct effect of these drugs on sweat gland cells (3). One may assume that early degenerative changes take place in the sweat glands after denervation.

Attempts to carry out analogous experiments on the cat by sciatic section or lumbar and sacral sympathetic extirpation were not successful because of the invariable development on the denervated foot pads of a thick dry horny layer which prevented an unequivocal comparison of the sweating capacities of the two sides.

#### *Pharmacological separation of pilomotor and sweat responses*

It has been found (1) that the pilomotor response to drugs with a nicotine-like action could be abolished in the cat's tail by intravenous administration of 3 mgm. of ergotamine tartrate. This was to be expected in view of the adrenergic nature of the pilomotor nerve fibers. In spite of total abolition of the pilomotor response by this method, the sweat response of the cat's paw remained unimpaired. On the other hand, atropinization of the cat abolished the sweat response to nicotine without affecting the pilomotor reaction. This also was to be expected in view of the cholinergic nature of the sudomotor fibers. When 1 mgm. of atropine was administered subcutaneously in man, the sweat response to nicotine was abolished but the pilomotor effect remained unimpaired.

#### *Local inhibition by drugs of sweat responses produced by heat and faradic stimulation*

In these experiments heat was applied by means of an electric hot box which covered the cat completely or which covered the trunk and arms of a human subject. Faradic stimulation was applied in threshold intensities to the sciatic nerve or to the sympathetic chain in the cat. Sweating elicited by these means could be checked in skin areas treated by injection of nicotine or apha-lobeline, or of local anesthetics. Thus a qualitative similarity was shown between the effect of these drugs on physiological sweat impulses and those caused by the local injection of nicotine. However, it was found that the impulses elicited by 1:200,000 nicotine were more sensitive to the paralyzing effect of these drugs than those elicited by electric stimulation or by heat stimuli.

Results of determinations of the minimum concentrations of these drugs which, if applied locally, inhibited sweat secretion following different stimuli,



were notable from two other standpoints. (a) Alpha-lobeline was more potent than nicotine in its nicotine-like action on sweat fibers. In man the optimum stimulating concentration of nicotine was 1:100,000, of alpha-lobeline 1:1,000,000; the minimum paralyzing concentration of nicotine was 1:5000, of alpha-lobeline 1:100,000. These figures indicate that alpha-lobeline is 10 to 20 times as potent as nicotine. By the blood pressure test the nicotine-like action of nicotine itself is stronger than that of alpha-lobeline. There is no explanation for this discrepancy at present. It was remarkable that 1:100,000 alpha-lobeline produced total inhibition of heat sweating in areas of human skin which were infiltrated intradermally with the drug. (b) Nerve fibers controlling the sweat response to nicotine were more sensitive to local anesthetics than were the sensory fibers. Cocaine, mixed in a concentration of 1:200,000 with an otherwise effective dose of nicotine, totally inhibited the sudomotor response. This concentration of cocaine has no apparent effect on sensory nerve fibers. This finding is in accord with the observation of Gasser and Erlanger (8) that the smaller the diameter of the nerve fiber the greater is its sensitivity to chemical anesthesia.

*Systemic effect of drugs with nicotine-like action on heat sweat*

Since the intradermal injection of adequate concentrations of drugs with nicotine-like action diminished locally the effect of any nervous sweat impulse, experiments were carried out to determine whether such anhidrotic effects could be obtained also by systemic administration of these drugs. Earlier observations (9) seemed to indicate that acetylcholine administered subcutaneously in relatively high doses might have some inhibitory influence on the sweat function in man.

The sweat gland activity before and at intervals after administration of the drug was tested in 4 different ways. (a) The insensible perspiration in human subjects was measured by the calcium chloride absorption method (10) at constant room temperature, before and after injection of acetylcholine or alpha-lobeline. (b) The time of onset, the intensity, and the extent of sweating produced by means of a heat chamber were noted in man before and after administration of these drugs. (c) The sweat responses to sciatic nerve stimulation by threshold intensities of the faradic current were observed before and after injection in the cat. (d) The local sweat responses to 1:100,000 nicotine were observed in man and in the cat before and after injection. No diminution of sweat gland activity could be seen by any of these methods after subcutaneous injection of 50 mgm. of acetylcholine or 20 mgm. of alpha-lobeline in man. In the cat highly toxic doses of acetylcholine, nicotine and alpha-lobeline administered subcutaneously and intravenously were without detectable effect on the sweat secretion.



## COMMENT

The analysis of the dual sweat response to intradermal injection of acetylcholine indicated that the sudden widespread response produced by concentrations ranging from 1:10,000 to 1:80,000 is due to the nicotine-like action of the drug. Identical responses were obtained by nicotine at an optimum concentration of 1:100,000 and by alpha-lobeline at an optimum concentration of 1:1,000,000, whereas these drugs did not show the direct muscarine-like action of acetylcholine on sweat glands. The abolition of the widespread response and the persistence of the more restricted effect of drugs acting directly on the glands after infiltration of the skin with high dilutions of local anesthetics, demonstrated the nervous nature of this response. Its independence of the central nervous system was proved by the fact that the response was unimpaired by fresh sectioning of the peripheral mixed nerve or by block anesthesia, and that it could be elicited in excised cat foot pads.

These results are interpreted as indicating that the local sweat response to intradermal nicotine occurs through an axon reflex mechanism. It must be assumed that, if the impulse arises at a point *s* (fig. 3, A) along the course of a fiber in the peripheral ramification of the sympathetic supply to the sweat glands, it proceeds from that point both efferently and antidromically. If the impulse arises at the nerve ending *s* (fig. 3, B), it runs only antidromically toward higher points of ramification, whence the impulse spreads efferently throughout an arborized peripheral distribution of the rami of the axon. The constant pattern of the response, if elicited repeatedly by injection at the same point, is explained by this mechanism. The response of small distant groups of sweat pores indicates single far reaching ramifications of the stimulated axons. Considering that the area of stimulation by intradermal injection does not exceed 0.5 cm. in diameter, and that the area of immediate response around the injection wheal is larger than 5 cm. in diameter, the phenomenon can hardly be interpreted in any other way.

Bickford (11) and Wilkins, Newman and Doupe (12) described a sweat response to faradic stimulation of the skin. This phenomenon was similar to the effect of drugs with nicotine-like action with respect to nerve degeneration, nerve block, and local anesthetics. These authors also interpreted their findings as an axon reflex. The question arises whether the faradic stimulation is effective by causing the liberation in the skin of acetylcholine which then, by virtue of its nicotine action, stimulates the sweat fibers, or whether it is a direct stimulation without chemical mediation.

In 1936 Lewis (13) described the spread of hyperalgesia around a spot of painful stimulation as an axon reflex in overlapping arborizations of axons supplying the region. He gave evidence against the existence of a continuous network of fibers by showing that a procaine wheal placed adjacent to the painful stimulus inhibited the spread of the hyperalgesia in its direction. If



the point of pain stimulus and the procaine wheal were in very close proximity, there was no extension of the hyperalgesia around the wheal as one would expect if the mechanism of spread were through a continuous network of nerve fibers. However, the hyperalgesia spread around the procaine wheal if the latter was made far enough from the injury so that the impulse might travel reflexly to points of ramification not blocked by the procaine.

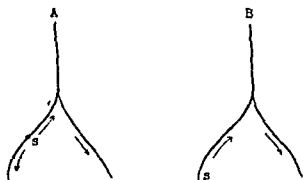


FIG. 3. DIAGRAM OF RAMIFIED SUDOMOTOR FIBERS, A AND B, STIMULATED BY NICOTINE AT POINTS s

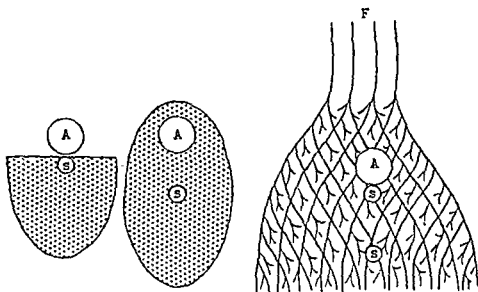


FIG. 4. DIAGRAM ILLUSTRATING HOW AXON REFLEXES OCCUR IN ARBORIZATIONS RATHER THAN IN NETWORKS OF PERIPHERAL NERVES

Shaded area = area of response; A = anesthetized area; s = point of stimulation; F = overlapping arborizations of nerve fibers.

Similar experiments were carried out in this laboratory using an intradermal nicotine injection with the ensuing pilomotor or sweat response, instead of a painful stimulation with its hyperalgesic response. The results suggested, in the same manner as did those of Lewis, that the spread of reaction occurs through an arborization rather than through a network of fibers. This is made clear by the diagram in figure 4.



It is of interest to note that in the sudomotor axon reflex set up by drugs with a nicotine-like action, the receptor point, whether that be the nerve ending or the fiber itself, behaves like an autonomic ganglion cell in that it is stimulated by small concentrations and paralyzed by large concentrations of such drugs. Thus, when the optimum stimulating concentration of acetylcholine is injected intradermally, the nicotine-like action of this drug sets up at the receptor point an impulse which travels to all connected nerve endings, where, because of the cholinergic nature of the sweat fibers, acetylcholine is liberated and stimulates the sweat glands into action by virtue of its muscarine-like action. An analogy to this course of events is seen in the physiological mechanism of sweating. Here also, acetylcholine operates at both ends of the postganglionic nerve fiber—at the ganglionic synapse by its nicotine-like action, and at the peripheral nerve ending by its muscarine-like action.

#### SUMMARY

1. Intradermal injection of high dilutions of drugs with a nicotine-like action (acetylcholine, nicotine, alpha-lobeline) caused an outbreak of sweat on the human skin in an area with a 2 to 5 cm. diameter around the wheal.

2. Intradermal acetylcholine produced sweating in two ways: directly by its muscarine-like action, and reflexly by its nicotine-like action.

3. Drugs with nicotine-like action also caused an outbreak of sweat droplets on the foot pad of the cat when injected into the pad.

4. This sweat response is abolished by infiltration of the skin with local anesthetics, remains active in areas anesthetized by nerve block, and can be elicited in extirpated cat's toe pads.

5. It is concluded that the sweating is effected through an axon reflex involving the terminal ramifications of the postganglionic sympathetic fibers supplying the sweat glands.

6. The nerve pathway over which this axon reflex occurs is 10 to 20 times as sensitive to local anesthetics as are sensory nerves.

7. The receptor point of the axon reflex behaves like an autonomic ganglion cell in being stimulated by small concentrations of drugs with nicotine-like action and paralyzed by large concentrations.

8. Attempts to demonstrate an anhidrotic effect of large doses of drugs with a nicotine-like action administered intravenously in cats or subcutaneously in man were unsuccessful.

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# PHYSICO-CHEMICAL PROPERTIES OF THE ARSPHENAMINES IN RELATION TO TOXICITY AND THERAPEUTIC EFFICIENCY<sup>1</sup>

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Received for publication April 25, 1941

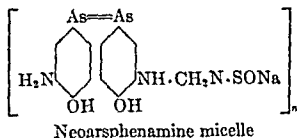
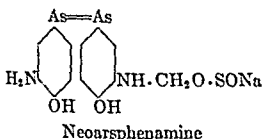
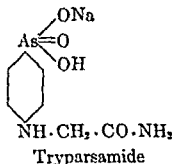
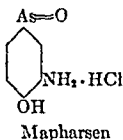
The organic arsenical compounds which possess only a single benzol ring, such as tryparsamide (Na *N*-phenyl-glycinamide-*p*-arsonate) and mapharsen (*3*-amino, *4*-hydroxy phenyl arsenious oxide) appear to behave in solution as typical crystalloid substances. The condensation of the *3*-amino, *4*-hydroxy phenyl arsenious oxide nucleus and its modifications to form the series of trivalent double ring compounds represented typically by arspenamine and its derivatives produces changes in the physical and chemical properties of these substances to an extent far greater than would be expected on the basis of the increase in the molecular weight alone, arspenamine dihydrochloride having a theoretical molecular weight of 475, disodium arspenamine one of 390, and neoarsphenamine one of 466. These changes in properties can be adequately explained only by the assumption that in the process of manufacture these compounds undergo aggregation of their molecules to the extent that at least a portion of these aggregates reaches a molecular size that places them in the realm of the colloids. The formulas of the compounds would therefore be more accurately expressed if written in such a way as to indicate this fact (see *neoarsphenamine micelle*).

In the case of solutions of emulsoid colloids such as the proteins the number of aggregated molecules "*n*" representing the micelle is usually a constant. In the case of the group of arspenamines, however, the number "*n*" appears not to be a constant but a variable representing the presence in the same solution of aggregates of widely different sizes. From the evidence available, particularly from dialysis experiments, it would appear that the range of

<sup>1</sup> A summary of the findings herein reported was read before the Minnesota Branch of the Society for Experimental Biology and Medicine (Proc. Soc. Exper. Biol. and Med. 1933, xxxi, 172, 175); before the Section on Medical Sciences of the American Association for the Advancement of Science, Minneapolis, June 27, 1935; and at a Mayo Foundation Exchange Lecture, Rochester, Minnesota, February 18, 1937.

A part of the funds used in this research was supplied by a grant from the Medical Research Fund of the University of Minnesota; a further portion was supplied by a research grant from E. R. Squibb and Sons, New York; and assistance in the preparation of materials was furnished by the personnel of the Civil Works Administration.





variability in the number of aggregated molecules comprising the different micelles must extend from particles having a molecular weight of less than 1000 (i.e.  $n = 1$  or  $2$ ) and which would be expected to behave as typical crystalloid particles, up to at least particles having a molecular weight in excess of 34,000 (i.e.  $n = 70-80$ ), or at least as large as molecules of egg albumin, and which would therefore be expected to behave as typical emulsoid colloids.

The colloidal characteristics of the arspenamines have been the subject of a number of investigations. Bauer (1) in 1919 investigated sodium arspenamine, silver arspenamine and atoxyl from this standpoint and concluded that these substances belonged to the class of so-called "semi-colloids", having particle sizes such as represent the dividing line between the colloids and crystalloids. Klemensiewicz (2) observed that the viscosity of aqueous solutions of arspenamine increased with the concentration and concluded that arspenamine is a lyophilic or emulsoid colloid, the greater viscosity of more concentrated solutions being due to progressive aggregation or polymerization of the compound as the concentration of the solution increased. More recently Voegtlin, Johnson and Dyer (3) found a direct relationship between the viscosity of acid and alkaline arspenamine solutions and their toxicity for rats; a decrease in viscosity on standing was associated with a great decrease in toxicity, a relation which was attributed to a progressive dispersion of the particles in solution, thereby decreasing the ability of such solutions to react with the colloidal constituents of the blood. They found that the method of manufacture greatly influenced the viscosity of the preparations, precipitation of the arspenamine hydrochloride with ether from methyl alcohol solution yielding preparations of lower viscosity and lesser toxicity than those precipitated with HCl from an aqueous solution. Sherndal (4) also studied the viscosity properties of a large number of samples of



arsphenamines prepared in various ways. He pointed out the gelatinous characteristics of arsphenamine especially in acid media and in the presence of ionized salts, and concluded that "arsphenamine is inherently a colloidal substance, and that electrolytes in certain concentrations produce coagulation of the disperse phase of its emulsoid sols." Raiziss and Gavron (5) investigated the colloidal properties of a few samples of arsphenamine and neoarsphenamine by dialysis using parchment membranes. Since these membranes are relatively impermeable only a small fraction of the arsenical passed through the membrane.

The great toxicity of arsphenamine dihydrochloride could readily be accounted for by the fact that in the form of its acid salt the arsphenamine aggregate would ionize as a positively charged colloid, whereas the blood proteins carry a negative charge. Mutual neutralization of the charges and intravascular precipitation of both colloids would therefore take place.

Lucké and Kolmer (6) observed early parenchymatous changes in the liver and kidneys of the lower animals as early as five hours after the injection of acid solutions of arsphenamine and stated that "under these circumstances immediate death is due primarily to acute vascular and intravascular changes of which congestion, thrombosis and embolism, especially of the lungs, are the prominent and sometimes sole lesions." Even the disodium salt, the form in which arsphenamine is injected clinically (or too frequently, from insufficient or inaccurate neutralization, the monosodium salt, which is much more colloidal) is insoluble at the pH of the blood. The insoluble arsphenamine base is undoubtedly formed when sodium arsphenamine is injected intravenously but, in the presence of a large excess of protein, the blood proteins probably peptize and redissolve the precipitated arsphenamine to form a colloidal solution of the arsphenamine base in a manner entirely analogous to the method employed in the commercial preparation of the soluble and colloidal protein silver preparations.

Attempts to demonstrate the precipitation *in vivo* of either the arsphenamine base or the plasma proteins have given negative results, except in the case of acid solutions of arsphenamine (Joseph (7), Schamberg, Raiziss, Weiss and Kolmer (8)). Such results, however, are to be expected in view of the powerful peptizing effects of the large excess of protein present in the blood. The inability to demonstrate macroscopic precipitation, however, in no way interferes with the proposition that "colloidal aggregates" are in large measure responsible for the immediate reactions (nitritoid crises) of flushing, dyspnea, precordial pain, cough, nausea, vomiting, and the symptoms of vasomotor collapse which sometimes occur shortly after intravenous injections of the arsphenamines. These effects could well be caused by changes in the physical state of the various colloidal systems, involving particularly changes in the state of hydration and aggregation of the proteins and the arsphenamines, without these changes becoming irreversible and proceeding to the point of actual precipitation.



Hirschfelder and Wright (9) studied the effects produced by neoarsphenamine and other lyophilic semi-colloids on solutions of egg albumin and rabbit plasma by means of the ultramicroscope and found evidences which suggested dehydration and aggregation of particles. The refractiveness of solutions of egg albumin was considerably increased following the addition of neoarsphenamine, indicating a change from the lyophilic toward the lyophobic state, a condition which normally precedes the precipitation of lyophilic colloids.

If the reasoning that a solution of any of the arsphenamines contains particles of all sizes, from the true crystalloid to the definitely colloid, is correct, it might be expected that particles of the same empiric chemical composition but of markedly different physical size would show differences in behavior both as regards toxic and therapeutic properties. Fundamentally a particle of definitely crystalloid size should not be capable of producing toxic effects of "anaphylactoid" or "nitritoid" origin through reaction with the blood proteins. Particles of crystalloid size might also be expected theoretically to be more active therapeutically than colloidal aggregates since they will probably ionize better and be more chemically reactive. The analogy may perhaps again be drawn between the crystalloid and colloid particles of the arsphenamines and of the silver salts. The inorganic silver nitrate is a crystalloid, strongly ionized and powerfully antiseptic. A protein silver preparation such as argyrol contains  $\frac{1}{3}$  as much silver as silver nitrate, but since 99 per cent of this silver is in the colloidal state and is non-ionized, the antiseptic properties are very much less than would be expected on the basis of its silver content.

Acting on the basis of these assumptions, we have endeavored to separate solutions of arsphenamine and neoarsphenamine into crystalloid and colloid fractions and to investigate the behavior of these two fractions in relation to their relative therapeutic and toxic effects.

#### METHODS AND MATERIALS

Experimentally the objective was to separate, from solutions of the arsphenamines, fractions which would represent (a) the micelles of lowest molecular weight in which "n" ranges from, say 1 to 10, that is, particles having molecular weights from 500 to 5000 approximately; and (b) the micelles of highest molecular weight in which "n" ranges from, say 60 to 80, that is, particles having molecular weights of 30,000 to 40,000.

This was accomplished through dialysis employing viscose (sausage casing) and cellophane (Dupont No. 300 and No. 600) membranes. The viscose membranes were considerably more permeable than those of cellophane, but were also more variable in their permeability. All of the membranes employed were, however, impermeable to egg albumin (M.W. 34,000) and to Congo Red. All membranes were thoroughly tested for leaks before use. Dialysis was carried out under a stream of purified nitrogen, a rapid stream being employed during the first hour to ensure complete elimination of oxygen from the system. All distilled water was freshly boiled and was cooled under nitrogen before use.

Preliminary tests showed that arsphenamine HCl may be maintained in aqueous



solution under nitrogen indefinitely without evidence of chemical deterioration, the iodine value (Wright (10)) remaining constant for 8 days and daily tests for arsenoxide (Rosenthal (11)) being invariably negative. No precipitation of the arsenical occurred.

When solutions of either alkalized arsphenamine (sodium arsphenamine) or neo-arsphenamine were dialyzed against water, it was found that the sodium ion diffused out and the arsenical precipitated within the dialyzing sac. Attempts to stabilize these solutions by dialysis against various buffered solutions containing the sodium ion were unsuccessful. Both arsenicals were found, however, to be perfectly stable when dissolved in and dialyzed against very dilute solutions (1:10,000) of sodium formaldehyde sulfoxylate adjusted to pH 9.0. The sodium formaldehyde sulfoxylate, however, had an appreciable peptizing effect on these arsenicals, increasing the size of the crystalloid fraction and decreasing the toxicity of the whole drug.

In order to avoid the formation of arsenoxide in the course of the dialysis it was found necessary to carry out the alkalization of arsphenamine HCl solutions under nitrogen within the dialyzing sac. Control solutions of the whole drugs were subjected to treatment identical with that of the fractions except that no membrane was employed.

The dialysate was removed at 24-hour intervals and replaced by fresh water or sodium formaldehyde sulfoxylate solution. The first 24-hour dialysate, kept under nitrogen until dialysis of the entire specimen was complete (6 days), was used as the "crystalloid fraction." The amount of arsenical passing through the membrane decreased rapidly in each succeeding 24-hour period, reaching a state after 4-5 days at which no more arsenical passed through the membrane. The portion of the arsenical remaining in the dialyzing sac after 6 days dialysis was used as the "colloid fraction." The intermediate fractions were preserved and analyzed to check the total recovery of arsenic which, in most instances was within 5 per cent of the theoretical.

Arsenic analyses of both crystalloid and colloid fractions, and of the intermediate collections, were made before the injection of the solutions employing the gravimetric method of Treadwell-Hall (12) recommended by Myers and du Mez (13) as a result of a comparative study of the accuracy of several methods of arsenic analysis. It was found impossible to employ the volumetric method of Lehmann (14) which is used for the U.S.P. assay of the arsphenamines (15), the oxidation with  $\text{KMnO}_4$  and  $\text{H}_2\text{SO}_4$  employed in this method being apparently inadequate to cause the complete breakdown of "aged" solutions of the arsphenamines, which become decidedly refractory to oxidation and almost invariably give low results with the Lehmann method.

The therapeutic and toxicity studies were all made on albino rats of the Wistar Institute strain, all animals being raised in our own colony and maintained on a constant fully adequate diet. For the therapeutic experiments animals weighing 150-200 Grams were inoculated 24 hours before the administration of the arsenical with approximately 300,000 organisms of *Trypanosoma equiperdum*.<sup>2</sup> At least two control animals were inoculated with the organism in each experiment. The virulence of the organism was maintained as constant as possible, so that untreated animals usually died on the 4th or 5th day. Test animals receiving injections of arsenical drugs were recorded as cured only after repeated microscopic examinations of the tail blood were negative for trypanosomes and the animals had survived for at least 30 days after the injection of the arsenical. For the toxicity studies animals weighing 80-120 grams were employed. Injections of the arsenical were made intravenously into the saphenous vein, a constant time of 2 minutes being employed for the injection. The volume of solution

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<sup>2</sup> The strain of *T. equiperdum* employed was kindly furnished to us by Dr. A. L. Tatum, Professor of Pharmacology, University of Wisconsin, to whom the authors desire to express their sincere thanks.



injected seldom exceeded 1 cc. Hemorrhage was controlled where necessary by ligation of the vein. Animals were recorded as having survived the injected dose if alive and apparently well after 14 days.

A total of more than 2500 rats was employed in the experiments reported in this paper.

### EXPERIMENTAL RESULTS

The experiments comprise a study of both the therapeutic and toxic effects of the crystalloid and colloid fractions separated from several samples (of different commercial brands) of neoarsphenamine, arsphenamine hydrochloride and alkalinized arsphenamine. The effects produced by solutions of the whole drug maintained under nitrogen for identical time periods were included for comparison.

#### *Therapeutic effects of crystalloid and colloid fractions of the arsphenamines*

Three brands of neoarsphenamine were used. Approximately 30 complete dialysis experiments with neoarsphenamine were made, of which 20 were complete and satisfactory in every detail, particularly as regards completeness of recovery of arsenic, absence of oxidation, and the employment of satisfactory dosage ranges for the injection of both series of animals. A total of 715 animals was employed in these twenty experiments, the results of which are summarized in table 1. The curative doses for the same three brands in the form of the whole drug subjected to treatment identical with that of the separated fractions were: Brand A, 10 mgm. per kilogram; Brand B, 12 mgm. per kilogram; Brand C, 15 mgm. per kilogram. The curative dose taken was the smallest dose at which all animals receiving that dose survived and above which no further deaths occurred.

The results presented in table 1 are arranged in order of decreasing therapeutic coefficient. It will be observed that in every case the crystalloid fraction was found to be more curative than the colloid fraction, the therapeutic coefficient ranging from 8.0 to 1.5 in the various experiments. In the 14 experiments with Brand A the crystalloid fraction was, on the average, 3.21 times more curative than the colloid fraction; Brand B averaged 2.96 times and Brand C 1.91 times more curative.

It may further be noted that the two highest therapeutic ratios of 8.0 and 5.14 are associated with the two sharpest separations of the crystalloid and colloid fractions, the colloid fractions in these instances being 14.37 and 22.01 times greater than the respective crystalloid fractions. Likewise the two lowest therapeutic ratios in the same series, namely of 1.50 and 1.65, are associated with the use of two very permeable membranes which brought about relatively poor separations of the two fractions, both dialysis ratios being less than 1.0.

This general agreement between the degree of separation of the smallest possible particles from those of largest molecular size, and the height of the therapeutic ratios may be illustrated in several ways. The 14 experiments reported with Brand A are all essentially repetitions of the same experiment.



The only variable was the unpredictable permeability of the viscose membranes employed in this series. The experiments are arranged in decreasing order of therapeutic ratios. Inspection of the table shows that in general the decline in the therapeutic ratios is accompanied by a similar decline in the dialysis ratios. In other words, the progressively larger proportion of the

TABLE 1

*Therapeutic ratios of colloid and crystalloid fractions of neoparsphenamine*

EXPERIMENT NUMBER	THERAPEUTIC EFFECT			DIALYSIS			DIALYSIS RATIOS	
	Colloid fractions	Crystalloid fractions	Colloid per crystalloid ratio	Colloid fractions	Colloid fractions	Colloid fractions	Colloid fractions	Colloid fractions
Brand A								
	mgm./kgm.	mgm./kgm.		mgm.	mgm.	mgm.		
58-9	24	3	8.00	219.8	15.3	48.3	14.37	4.55
76-7	36	7	5.14	416.0	18.9	103.4	22.01	4.02
24-5	18	4	4.50	449.0	45.1	135.9	9.96	3.30
74-5	34	9	3.78	357.0	145.0	257.0	2.46	1.39
30-1	18	5	3.60	512.7	48.2	96.7	10.64	5.30
72-3	26	8	3.25	337.8	122.0	242.7	2.77	1.39
56-7	26	9	2.89	205.8	55.7	85.9	3.69	2.40
32-3	14	5	2.80	450.3	76.1	135.9	5.92	3.31
16-7	18	8	2.25	329.7	74.5	159.0	4.43	2.07
46-7	26	13	2.00	218.9	221.8	336.8	0.99	0.65
60-1	20	11	1.82	286.7	174.4	320.9	1.64	0.89
48-9	24	14	1.71	168.0	176.0	379.0	0.95	0.44
36-7	18	11	1.64	287.4	206.6	319.1	1.39	0.90
34-5	18	12	1.50	218.3	323.0	403.0	0.68	0.54
Brand B								
66-7	22	6	3.67	299.0	130.0	326.5	2.30	0.92
68-9	24	7	3.43	505.5	35.9	147.9	14.08	3.42
64-5	16	9	1.78	208.3	310.2	385.7	0.67	0.54
Brand C								
42-3	20	9	2.22	246.3	182.7	219.2	1.35	1.12
44-5	18	9	2.00	233.9	178.5	188.5	1.31	1.24
38-9	18	12	1.50	125.1	323.9	325.8	0.39	0.38

drug found in the crystalloid fraction, due to the use of increasingly permeable membranes, and consequently poorer separation of the semi-colloid from the crystalloid material, finds expression in progressively lower therapeutic ratios for the crystalloid fraction.

Statistical comparison of the degree of correlation between the therapeutic and dialysis ratios gives a correlation coefficient of 0.784 for the correlation of



the therapeutic and 24 hour dialysate ratios and 0.765 for the correlation of the therapeutic and total dialysate ratios, indicating a relatively high degree of correlation between the therapeutic and dialysis ratios.

TABLE 2

*Therapeutic ratios of colloid and crystalloid fractions of arspenamine dihydrochloride and alkalinized arspenamine*

EXPERIMENT NUMBER	THERAPEUTIC EFFECT			DIALYSIS			DIALYSIS RATIO	
	Colloid fractions	Crystalloid fractions	Colloid per crystalloid ratio	Colloid fraction	Crystalloid fractions, 24 hours	Crystalloid fractions total	Colloid per crystalloid, 24 hours	Colloid per crystalloid total
Arsphenamine hydrochloride								
Brand D								
	mgm./kgm.	mgm./kgm.		mgm.	mgm.	mgm.		
102-3	7.5	1.0	7.50	75.1	45.5	88.6	1.65	0.85
104-5	7.0	2.0	3.50	84.6	64.7	108.4	1.31	0.78
Brand E								
109-10	6.5	1.5	4.33	220.3	76.0	126.7	2.90	1.74
113-14	7.0	2.0	3.50	77.6	94.8	122.4	0.82	0.63
Alkalinized arspenamine								
Brand E								
126-7	12.0	2.5	4.80	322.2	103.9	265.2	3.10	1.21
130-1	8.0	2.5	3.20	32.8	38.1	189.6	0.86	0.17
Brand F								
141-2	11.0	2.5	4.40	185.0	87.6	147.0	2.11	1.26
143-4	11.0	3.0	3.67	160.5	95.7	182.4	1.68	0.88

It may further be noted that the mean therapeutic ratios for the three brands likewise follow the order of the dialysis ratios for the corresponding brands.

BRAND	MEAN RATIOS	
	Therapeutic	Dialysis 24 hours
A	3.21	5.85
B	2.96	5.68
C	1.91	1.02

A smaller number of experiments were carried out on arspenamine. A total of 640 animals was used in the eight experiments reported in table 2, and in the determination of the curative doses of the whole drugs.



The curative doses of the three brands of arsphenamine employed in the form of the whole drug subjected to treatment identical with that of the separated portions were: Brand D, arsphenamine HCl 5.5 mgm. per kilogram; Brand E, arsphenamine HCl 3.5 mgm. per kilogram, alkalized 4.0 mgm. per kilogram; Brand F, alkalized 4.0 mgm. per kilogram.

The results presented in table 2 for arsphenamine show that in both the acid and alkalized forms essentially the same situation applies as was found in the case of neoarsphenamine, namely (1) that the crystalloid fractions were invariably more curative than the colloid fractions, the curative ratios ranging from 7.5 to 3.5 for acid arsphenamine and from 4.8 to 3.2 for alkalized arsphenamine and (2) that the more sharply cut the separation of the crystalloid and colloid fractions the greater is the therapeutic ratio, i.e. the more highly curative is the crystalloid fraction which ranged from 1.33 to 5.5 times the curative activity of the whole drug.

*Toxic effects of crystalloid and colloid fractions of the arsphenamines*

Five samples of three brands of neoarsphenamine were employed in these experiments. A total of 634 animals was employed, 279 for the determinations of the toxicity of the whole drug and 355 for the toxicity of the separated fractions. The values for L.D. 50 were calculated by the double integration method of Dragstedt and Lang (16) and Behrens (17).

The values obtained for the L.D. 50's of the whole drug of the five samples of neoarsphenamine were:

	<i>mgm./kgm.</i>
Brand A, Lot 1.....	357.5
Lot 2'.....	387.5
Brand B. ....	500.0
Brand G, Lot 1.....	508.5
Lot 2 .....	318.5

The results of the separation of the crystalloid and colloid fractions by dialysis and the values obtained for the respective L.D. 50's are given in table 3. From this it will be observed that (1) in every case the crystalloid fraction was much less toxic than the colloid fraction, the enhanced toxicity of the colloid fraction ranging from 1.54 to 4.41 (mean 2.81) times that of the crystalloid fraction and (2) with one exception (Brand G, Lot 1) the crystalloid fraction was less toxic than the corresponding whole drug.

A similar series of experiments was likewise carried out with arsphenamine both in the form of the hydrochloride and after alkalization with sodium hydroxide. Three brands of arsphenamine were employed in these experiments. A total of 512 animals was used, 182 being for the determination of the toxicity of the whole drug and 320 for the toxicity of the fractions.



The values obtained for the L.D. 50's of the three brands of arspenamine were:

	mgm./kgm.
Brand D, as hydrochloride.....	39.5
Brand E, as hydrochloride.....	37.0
as sodium salt.....	112.5
Brand F, as sodium salt.....	193.0

The analytical results of the separation of the crystalloid and colloid fractions and their corresponding toxicities are given in table 4. The results obtained with arspenamine show even more strikingly than was the case with neoarsphenamine that the crystalloid fraction was invariably (1) much less

TABLE 3  
*Toxicity ratios of colloid and crystalloid fractions of neoarsphenamine*

EXPERIMENT NUMBER	TOXIC EFFECT			DIALYSIS			DIALYSIS RATIOS	
	Colloid fractions, L.D. 50	Crystal- loid fractions, L.D. 50	Colloid per crystalloid ratio	Colloid fractions	Crystal- loid fractions, 24 hours	Crystal- loid fractions total	Colloid per crys- talloid, 24 hours	Colloid per crys- talloid total
Brand A								
	mgm./ kgm.	mgm./ kgm.		mgm.	mgm.	mgm.		
202-3 (lot 1)	237.5	437.5	1.89	1024.0	616.0	959.0	1.66	1.07
207-8 (lot 2)	272.0	417.5	1.54	1500.0	450.0	640.0	3.33	2.34
Brand B								
212-3	187.5	550.0	2.93	1390.0	650.0	1607.5	2.14	0.80
216-7	175.0	513.0	2.98	1215.0	672.5	1438.5	1.81	0.84
Brand G								
222-3 (lot 1)	162.0	500.0	3.09	642.0	1526.0	2376.0	0.42	0.27
225-6 (lot 2)	85.0	375.0	4.41	930.0	710.0	1180.0	1.31	0.80

toxic than the colloid fraction and also (2) appreciably less toxic than the whole drug. The greater toxicity of the colloid fraction as compared with that of the crystalloid fraction ranged from 2.88 to 9.07 (mean 5.20) times for arspenamine hydrochloride and from 1.86 to 3.53 (mean 2.66) times for the alkalinized arspenamine.

The differences in the type of toxic reaction produced by the crystalloid and colloid fractions were, however, even more striking than the differences in dosage. The same type of phenomena occurred with all three groups of preparations employed, namely, neoarsphenamine, arspenamine hydrochloride and alkalinized arspenamine.

Following the injection of the crystalloid fractions from all three groups of



preparations the animals invariably died a relatively delayed death following the typical symptoms of arsenical poisoning such as are usually seen in routine toxicity studies with the whole drug. The animals died over a period of 1 to 8 days, the average duration of life after injection being 62 hours.

Following the injection of the colloid fraction, however, more than 85 per cent of the animals that died did so within  $\frac{1}{4}$  to 3 hours of injection. The injection of the colloid fraction was followed almost immediately by an acute

TABLE 4

*Toxicity ratios of colloid and crystalloid fractions of arsphenamine dihydrochloride and alkalinized arsphenamine*

EXPERIMENT NUMBER	TOXIC EFFECT			DIALYSIS			DIALYSIS RATIOS	
	Colloid fractions, L.D. 50	Crystalloid fractions, L.D. 50	Colloid per crystalloid ratio	Colloid fractions	Crystalloid fractions, 24 hours	Crystalloid fractions, total	Colloid per crystalloid, 24 hours	Colloid per crystalloid total
Arsphenamine hydrochloride								
Brand D								
	mgm./kgm.	mgm./kgm.		mgm.	mgm.	mgm.		
252-3	7.5	68.0	9.07	110.0	72.5	89.2	1.50	1.23
256-7	12.5	72.0	5.98	225.0	165.4	218.6	1.36	1.03
Brand E								
262-3	21.5	62.5	2.91	259.0	91.7	194.8	2.83	1.33
264-5	20.0	57.5	2.88	256.0	95.1	124.3	2.69	2.05
Alkalinized arsphenamine								
Brand E								
277-8	65.0	133.5	2.06	665.9	142.2	264.8	4.68	2.50
281-2	67.5	125.0	1.86	598.4	256.0	372.5	2.34	1.61
Brand F								
287-8	63.0	222.5	3.53	422.5	198.0	256.5	2.13	1.65
289-90	75.0	237.5	3.17	398.0	215.5	286.0	1.84	1.39

reaction in which the animals showed extreme respiratory embarrassment, exudation of a serosanguinous fluid from the nose and mouth, and death by respiratory failure. Similar reactions occurred from sublethal doses but were followed by recovery. The animals that died usually did so within 30 minutes and those that survived more than 3 hours usually survived indefinitely since the dose which produced these acute toxic reactions was in almost all instances much smaller than that which was capable of producing a delayed death from arsenical poisoning.

#### *Therapeutic indices*

In tables 1 to 4 the therapeutic and toxicity ratios were calculated for the relationship between the corresponding crystalloid and colloid fractions



In table 5 the therapeutic and toxicity ratios are presented calculated in relationship to the corresponding whole drugs. The values entered in the table are the mean values for all experiments reported with each preparation. The therapeutic index is calculated as the product of the therapeutic and toxic ratios, and therefore is an expression of the superiority or inferiority of any particular preparation with reference to the whole drug from the combined

TABLE 5

*Therapeutic indices of the crystalloid and colloid fractions of neoarsphenamine and arsphenamine compared to the whole drug*

DRUG AND FRACTION	THERAPEUTIC RATIO		TOXICITY RATIO		THERAPEUTIC INDEX	
Neoarsphenamine						
Brand A						
Crystalloid fraction . . . .	1.18		1 22		1.44	
Colloid fraction . . . . .		0.43		0.67		0.29
Brand B						
Crystalloid fraction.... .	1.65		1.10		1.82	
Colloid fraction .... .		0.57		0.37		0.21
Arsphenamine HCl						
Brand D						
Crystalloid fraction... .	3.67		1 76		6.44	
Colloid fraction..... .		0.75		0.24		0.18
Brand E						
Crystalloid fraction... .	2.00		1 62		3 24	
Colloid fraction..... .		0.52		0 56		0 29
Alkalinized arsphenamine						
Brand E						
Crystalloid fraction... .	1.60		1 15		1.84	
Colloid fraction. .... .		0.40		0.59		0.24
Brand F						
Crystalloid fraction..... .	1.45		1.20		1.74	
Colloid fraction..... .		0.36		0 31		0.11

standpoints of curative and toxic properties. The therapeutic index of all specimens of the whole drug is therefore 1.

That is,

$$\text{Therapeutic ratio} = \frac{\text{Curative dose of whole drug}}{\text{Curative dose of fraction}}$$

$$\text{Toxicity ratio} = \frac{\text{L.D. 50 dose of fraction}}{\text{L.D. 50 dose of whole drug}}$$

$$\text{Therapeutic index} = \text{Therapeutic ratio} \times \text{toxicity ratio}$$



It may be observed from the table that the therapeutic ratio, toxicity ratio, and therapeutic index of every crystalloid fraction is greater than 1, while the corresponding value of every colloid fraction is less than 1, and in the case of the therapeutic index indicate an efficiency as compared with the whole drug of only 10 to 30 per cent.

#### DISCUSSION

The experiments reported above confirm the fact that both arsphenamine and neoarsphenamine possess decided colloidal characteristics, and show that there is considerable difference in the quantitative relationships of these colloidal characteristics not only between different brands of the same drug but even between different lots of the same brand.

The dialysis experiments with their membranes of varied permeability show conclusively the correctness of the assumption made in the introductory discussion that solutions of the arsphenamines contain particles in all stages of aggregation from the molecular range (M. W. under 500) up to aggregates composed of more than 80 molecules and having molecular weights in excess of 35,000 (i.e., of the order of magnitude of egg albumin).

These colloidal particles appear to possess properties which are decidedly disadvantageous from the standpoint of the patient receiving antisyphilitic medication since they are less curative, more toxic and are responsible for the immediate toxic reactions. The fact that these reactions were most intense and occurred from the smallest dosage with arsphenamine hydrochloride in which the colloidal aggregates would carry a charge opposite in sign to that of colloidal blood proteins indicates that these immediate toxic reactions are in the nature of colloidoclastic reactions produced by still further aggregation of colloidal particles following the injection of the drug.

The fact that samples of the crystalloid fraction of neoarsphenamine were obtained having a curative dose of 3.0 mgm. per kilogram and of alkalinized arsphenamine of 2.5 mgm. per kilogram is of considerable significance in the light of Rosenthal's (11) finding that both arsphenamine and neoarsphenamine are at least partly converted into arsenoxide in the body after injection. Tatum and Cooper (18) found the curative dose of mapharsen corresponding to that which we employed for the arsphenamines (i.e., the smallest dose giving 100 per cent cures) to be 1.2 mgm. per kilogram. Data shortly to be published from this laboratory confirm this value. It is evident, therefore, that adequate suppression of the colloidal properties of the arsphenamines in the course of manufacture is capable of yielding preparations of these drugs having a curative value on a weight for weight basis of at least one-half that of mapharsen rather than the value of one-tenth to one-fifteenth usually assigned to them.



## CONCLUSIONS

1. Solutions of neoarsphenamine, arsphenamine hydrochloride and 'alkalinized' arsphenamine have been separated into relatively crystalloid and colloid fractions by means of dialysis in an inert atmosphere, and the therapeutic and toxic properties of these fractions have been investigated.

2. The crystalloid fractions of neoarsphenamine and arsphenamine (both acid and alkalinized) are more curative than either the whole drug or the colloidal fraction. Crystalloid fractions of neoarsphenamine were up to 8.0 times as curative as the colloidal fraction and 3.3 times as curative as the whole drug; of acid arsphenamine 7.5 times and 5.5 times as curative, and of alkalinized arsphenamine 4.8 times and 1.6 times as curative respectively.

3. The crystalloid fractions of neoarsphenamine and arsphenamine (both acid and alkalinized) were less toxic than either the colloid fraction or the whole drug. Crystalloid fractions of neoarsphenamine were up to 4.4 times less toxic than the colloid fraction and 1.2 times less toxic than the whole drug; of acid arsphenamine 9.1 times and 1.8 times, and of alkalinized arsphenamine 3.5 and 1.2 times respectively.

4. The crystalloid fractions of neoarsphenamine and arsphenamine (both acid and alkalinized) produced death following the typical symptoms of arsenical poisoning, the average duration of life after injection being 62 hours. The colloid fractions of neoarsphenamine and arsphenamine (both acid and alkalinized) produced immediate toxic reactions resulting in death from respiratory failure within one-half to 3 hours in more than 85 per cent of all deaths.

5. The therapeutic and toxicity ratios and therapeutic indices of all specimens studied have been calculated in terms of the respective whole drugs (table 5).

6. The desirable properties of high curative index and low toxic index appear to reside in the least aggregated portion of the arsphenamines. The strongly aggregated colloidal portion appears to possess only the undesirable properties of low curative index and high toxic index, producing immediate toxic reactions after injection in small dosage.

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# THE LOCAL ANESTHETIC ACTIVITY OF CERTAIN DERIVATIVES OF $\alpha$ AND $\beta$ -NAPHTHOL<sup>1,2</sup>

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Received for publication May 6, 1941

The naphthalene nucleus, as compared with the benzene nucleus, has been utilized only infrequently in the preparation of local anesthetics. A number of naphthoates were found to be anesthetic by Fisk and Underhill (1) but inapplicable clinically because they produced irritation. Sergievskaya and Nesvadba (2) report local anesthetic effects for certain esters of 4-aminonaphthoic acid and Rowe (3) states that of 22 modifications of this nucleus the  $\beta$ -diethylaminoethyl ester (naphthocaine) was the most outstanding. Haury, Gruber and Drake (4) extended the observations of Rowe (3) and reported naphthocaine to be equally toxic but ten times more active on rabbit cornea than cocaine. A number of new naphthol derivatives have been made available<sup>3</sup> to us (table 1). Compounds I and II are morpholine and piperidine derivatives of  $\beta$ -naphthol; V and VII are aminobenzoates of I and II whereas III and IV are aminobenzoates of morpholine and piperidine modifications of  $\alpha$ -naphthol. Compound VI is a permutation of VII and differs from the latter in that it is a benzoate and not an aminobenzoate. The present studies were designed to determine if the naphthol derivatives produced local anesthesia, and to compare them with procaine and cocaine.

## DERMAL ANESTHESIA

The technique described by Rose (5) was used to detect anesthesia. Aqueous solutions of the compounds were injected in 0.1 cc. volumes intradermally in guinea-pigs shaved free of hair and the presence or absence of anesthesia determined by faradic stimulation of each area injected. Procaine served as the standard of comparison for duration of anesthesia.

*Procaine.* In the present experiments procaine in 0.1, 0.5 and 1.0 per cent

<sup>1</sup> This investigation was supported in part by the D. J. McCarthy Foundation and the Smith, Kline and French Laboratories Fellowship.

<sup>2</sup> Presented before the Society for Pharmacology and Experimental Therapeutics, Chicago, April, 1941.

<sup>3</sup> These compounds have been made available to us by Dr. R. L. Shriner of the Department of Organic Chemistry of the University of Illinois.



TABLE 1

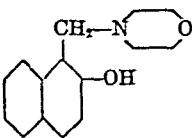
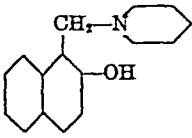
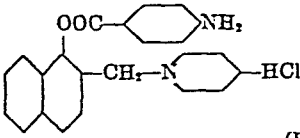
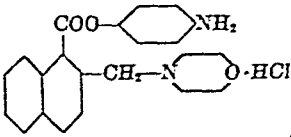
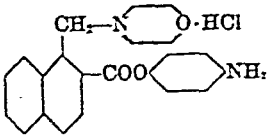
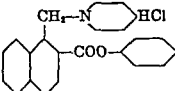
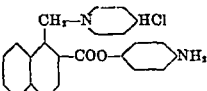
NAME	FORMULA	M.P.	DURATION OF ANESTHESIA IN GUINEA-PIGS			
			Without epinephrine			With epinephrine
			0.1%	0.5%	1.0%	0.1%
			Minutes			Minutes
Diethyl-amino-ethyl para-aminobenzoate hydrochloride	$\text{H}_2\text{N}-\text{C}_6\text{H}_4-\text{COO}(\text{CH}_2)_2\text{N}(\text{C}_2\text{H}_5)_2$ <p>Procaine</p>	°C.	2	16	29	53
α-morpholino-methyl-β-naphthol	 <p>(I)</p>	115-116	0	10	49	5
α-piperidino-methyl-β-naphthol	 <p>(II)</p>	94-95	6	28	67	18
β-piperidino-methyl α-naphthyl p-aminobenzoate HCl	 <p>(III)</p>	76.5-79.5	19			112
β-morpholino-methyl α-naphthyl p-aminobenzoate HCl	 <p>(IV)</p>	191-192				
α-morpholino-methyl β-naphthyl p-aminobenzoate HCl	 <p>(V)</p>	181-183				



TABLE 1—Continued

NAME	FORMULA	M.P.	DURATION OF ANESTHESIA IN GUINEA-PIGS			
			Without epinephrine			With epinephrine
			0.1%	0.5%	1.0%	0.1%
			Minutes			Minutes
$\alpha$ -piperidino-methyl $\beta$ -naphthyl benzoate HCl	 (VI)	°C. 185-186	23	38	88	104
$\alpha$ -piperidino-methyl $\beta$ -naphthyl <i>p</i> -amino-benzoate HCl	 (VII)	151-153	21	42	129	165

concentrations produced anesthesia by intracutaneous injection in guinea-pigs for an average of 2, 16 and 29 minutes (table 1).

In 1903 Braun (6) discovered that epinephrine intensified and prolonged the action of anesthetics. In the present studies 0.1, 0.5 and 1.0 per cent procaine in 1:100,000 epinephrine produced anesthesia for 53, 74, and 89 minutes.

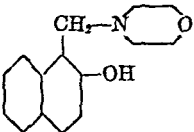
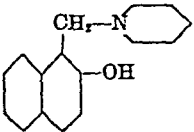
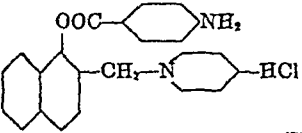
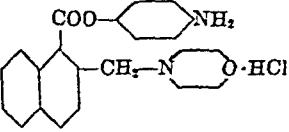
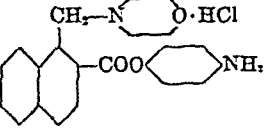
*Compound I.* This compound failed to produce anesthesia after intradermal injection in 0.1 but in 0.5 and 1.0 per cent concentration anesthesia was observed for 10 and 49 minutes respectively (table 1). In 0.1 and 0.5 per cent solution in 1:100,000 epinephrine, compound I caused anesthesia for 5 and 29 minutes.

*Compound II.* In 0.1, 0.5 and 1.0 per cent solution II produced anesthesia for 6, 28 and 67 minutes. Augmentation of the activity of II by epinephrine is indicated by the fact that in 0.1 and 0.5 per cent concentrations in 1:100,000 epinephrine, anesthesia was observed for 18 and 54 minutes.

*Compound III.* In 0.1 per cent solution (slight cloudiness) III produced anesthesia intradermally in guinea-pigs for 19 minutes. In 1:100,000 epinephrine 0.1 per cent solution of III caused anesthesia for 112 minutes



TABLE 1

NAME	FORMULA	M.P.	DURATION OF ANESTHESIA IN GUINEA-PIGS				
			Without epinephrine			With epinephrine	
			0.1%	0.5%	1.0%	0.1%	0.1%
			Minutes			Minutes	
Diethyl-amino-ethyl para-aminobenzoate hydrochloride	$\text{H}_2\text{N}-\text{C}_6\text{H}_4-\text{COO}(\text{CH}_2)_2\text{N}(\text{C}_2\text{H}_5)_2\text{Cl}$ <p>Procaine</p>	°C.	2	16	29	53	
$\alpha$ -morpholino-methyl- $\beta$ -naphthol	 <p>(I)</p>	115-116	0	10	49	5	
$\alpha$ -piperidino-methyl- $\beta$ -naphthol	 <p>(II)</p>	94-95	6	28	67	18	
$\beta$ -piperidino-methyl $\alpha$ -naphthyl <i>p</i> -amino-benzoate HCl	 <p>(III)</p>	76.5-79.5	19			112	
$\beta$ -morpholino-methyl $\alpha$ -naphthyl <i>p</i> -amino-benzoate HCl	 <p>(IV)</p>	191-192					
$\alpha$ -morpholino-methyl $\beta$ -naphthyl <i>p</i> -amino-benzoate HCl	 <p>(V)</p>	181-183					



Anesthesia was detected by touching the cornea with the rounded end of a fine glass rod, absence of the winking reflex being the criterion. It was found that the average duration of anesthesia after 1.0 per cent cocaine was 20 minutes. Compounds I, II, VI, and VII produced anesthesia for an average of 9, 20, 12 and 19 minutes respectively (table 2).

#### TOXICITY

The M.L.D. of procaine after subcutaneous injection in guinea-pigs in the present studies was 375 while that of I was above 400 and that of II approximately 250 mgm. per kilogram of body weight (table 2). In doses of from 700 to 1000 mgm. per kilo VII failed to cause an acute death in the guinea-pigs injected although several deaths were noted over night in the 900 and 1000 mgm. dose range.

#### DISCUSSION

While it was found in the present studies that compounds I, II, III, VI and VII produced marked anesthesia in guinea-pigs by intradermal injection they are inferior to procaine because all produce tissue damage. The marked dermal anesthetic activity and extremely low subcutaneous toxicity of VII gave promise of valuable anesthetic properties, but any attempt to modify the irritant properties of its solutions resulted in precipitation of the free base. In 1.0 per cent solution compounds I, II and VI produced anesthesia of poor depth and also caused irritation when instilled in a rabbit's eye. After topical application VII did not appear irritating and produced anesthesia of good depth in 1.0 per cent solution but in higher concentrations irritation was apparent and neither depth nor duration of anesthesia was materially increased. The present naphthol derivatives are, therefore, inferior to procaine and cocaine as local anesthetics.

#### SUMMARY

1. In the present experiments the hydrochlorides of  $\alpha$ -morpholino methyl- $\beta$  naphthol (I),  $\alpha$ -piperidino methyl- $\beta$ -naphthol (II),  $\beta$ -piperidino methyl  $\alpha$ -naphthyl p-aminobenzoate (III),  $\beta$ -morpholino methyl  $\alpha$ -naphthyl p-aminobenzoate (IV),  $\alpha$ -morpholino methyl  $\beta$ -naphthyl p-aminobenzoate (V),  $\alpha$ -piperidino-methyl  $\beta$ -naphthyl benzoate (VI) and  $\alpha$ -piperidino methyl- $\beta$ -naphthyl p-aminobenzoate (VII) were found to produce local anesthesia.

2. Compounds I and II produced marked anesthesia intracutaneously in guinea-pigs but caused irritation. Compound II also was more toxic than procaine subcutaneously in guinea pigs. Compounds III, IV and V are very unstable in solution and produce anesthesia of poor depth and variable duration.

3. Compounds VI and VII produced marked anesthesia and VII was found



to have an extremely low subcutaneous toxicity. Both compounds produced irritation in corneal and intradermal tests.

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# THE RELATION OF THE LIVER HISTAMINE TO ANAPHYLACTIC SHOCK IN DOGS

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Received for publication May 12, 1941

Dragstedt and Gebauer-Fuelnegg (1), Dragstedt and Mead (2), and Code (3), have demonstrated that histamine is liberated into the blood during acute anaphylactic shock in dogs. Dragstedt and Mead estimated the amount of histamine liberated by comparing the time for its disappearance from the blood with the times for the disappearance of known doses of histamine. Code made quantitative determinations of the histamine in the blood of shocked animals and in the blood of animals that had received known doses of histamine. The conclusion was drawn in both cases that the amount of histamine liberated during an anaphylactic reaction is adequate to account for the vascular effects observed.

There is a considerable amount of evidence which indicates that the liver is of prime importance in the anaphylactic reaction of the dog and that, correspondingly, the liver should be the chief tissue from which the histamine is liberated during the reaction. Manwaring (4) reported that ligation of the aorta and vena cava above the diaphragm prevented the development of anaphylaxis in dogs and concluded that some infra-diaphragmatic organ was essential to its occurrence. As removal of the intestines, stomach, kidneys, adrenals and spleen had no effect, while short-circuiting the liver out of the circulation did prevent the reaction, he concluded that the liver was the essential organ. That the integrity of the liver is necessary for the development of the complete symptomatology of anaphylactic shock in the dog is supported by the observations of Voegtlin and Bernheim (5), who failed to induce anaphylactic shock in dogs after the production of an Eck fistula and ligation of the hepatic artery, by Denecke (6) with similar experiments, and by Simonds and Brandes (7) who found that anaphylactic shock did not occur in dogs when the antigen was injected while the circulation through the liver was prevented by occlusion of the hepatic veins, but that restoration of the circulation through the liver permitted the reaction to develop. Recently, however, Waters and Markowitz (8) have reported the occurrence of "typical anaphylaxis" in dogs after a hepatectomy. Although one could properly question the justification for employing the term "typical ana-



phylaxis" upon the evidence which they (9) submitted that hepatectomy prevents the typical change in blood coagulation which occurs during anaphylaxis, nevertheless their demonstration of a fall in blood pressure following the injection of serum in serum-sensitized hepatectomized dogs prompted a reinvestigation of the rôle of the liver in anaphylaxis, with particular reference to its behavior as a source of histamine. The early observations of Dragstedt and Gebauer-Fuelnegg, that the histamine activity of blood drawn from the vena cava above the entrance of the hepatic veins was greater than that of blood drawn from the portal vein, were consistent with the assumption that the liver is an important source of the liberated histamine. Watanabe (10) reported that the liver histamine of recently shocked dogs was much less than that of sensitized animals. As only a small number of animals were studied, however, and as the liver histamine varies considerably from animal

TABLE 1  
*Liver histamine of normal dogs during ether anesthesia*

DOG	WEIGHT	LIVER WEIGHT	RATIO LIVER WEIGHT TO BODY WEIGHT	INITIAL LIVER HISTAMINE (APPROXIMATELY 10 MINUTES ANESTHESIA)		LIVER HISTAMINE AFTER 40 MINUTES ANESTHESIA	
				Micrograms per gram	Total milligrams	Micrograms per gram	Total milligrams
	<i>kilos</i>	<i>grams</i>	<i>per cent</i>				
1	10.0	370	3.7	37.5	13.8	37.5	13.8
2	13.0	380	2.9	37.5	14.2	50.0	19.0
3	10.0	450	4.5	75.0	33.7	75.0	33.7
4	10.5	250	2.3	35.0	8.7	35.0	8.7
5	7.2	345	4.8	150.0	51.7	150.0	51.7
6	9.0	288	3.2	120.0	34.5	120.0	34.5

All histamine values in terms of histamine acid phosphate.

to animal, these findings can only be considered as suggestive. The experiments here reported were therefore performed, in which the liver histamine was determined before and after shock in each animal, in order that the amount of histamine liberated from the liver could be correlated with the degree of shock and a measure of the rôle of the liver determined thereby.

The dogs were sensitized by the injection of 10 cc. of horse serum, 5 cc. intravenously and 5 cc. subcutaneously. Two weeks later the animals were weighed, anesthetized with ether, and arranged for a blood-pressure record. The abdomen was opened, and a small piece of liver (5 to 10 grams) removed, washed in saline, and weighed. Care was taken to arrest bleeding. The shocking dose of serum (2 cc. per kilo) was injected *via* the femoral vein. About twenty minutes later a second piece of liver was obtained. The liver samples were extracted by Best's method (11) and assayed for their histamine content against standard solutions of histamine on the blood pressure of the



etherized, atropinized cat. The liver was removed, drained of blood, and weighed. The total pre-shock and post-shock liver histamine values were then computed. Control experiments on normal animals indicate that there is no decrease in the liver histamine incident to the experimental procedures (see table 1). In the shocked animals a factor that might influence the

TABLE 2

*Liver histamine before and after anaphylactic shock in dogs*

DOG	WEIGHT	DEGREE OF SHOCK*	LIVER WEIGHT	RATIO LIVER WEIGHT TO BODY WEIGHT	INITIAL LIVER HISTAMINE		POST SHOCK LIVER HISTAMINE		HISTAMINE LOST FROM LIVER	
					Micro-grams per gram	Total milli-grams	Micro-grams per gram	Total milli-grams	Total milli-grams	Mgm./kilo of dog
	kilos		grams	per cent						
1	9	0	382	4.2	120	45.84	120	45.84	0	0
2	16	0	366	2.3	30	10.98	30	10.98	0	0
3	9.8	+	324	3.3	50	16.2	60	19.4	Gain	
4	20	+	574	2.8	90	51.66	75	43.05	8.61	0.43
5	8	+	221	2.8	60	13.26	45	9.95	3.31	0.41
6	16	++C	532	3.3	85	45.22	65	34.58	10.6	0.66
7	8	++B	269	3.3	90	24.20	45	12.10	12.10	1.50
8	11.4	++B	420	3.6	90	37.8	60	25.2	12.6	1.1
9	13	++B	426	3.3	71	30.3	23	9.8	20.5	1.6
10	17.3	++B	495	2.8	150	74.25	75	37.13	37.1	2.1
11	7.7	++A	330	4.3	60	19.8	6	2.0	17.8	2.3
12	11	+++	539	4.9	75	40.4	21	11.3	29.1	2.6
13	11.8	+++	443	3.7	165	73.1	35	15.5	57.5	4.8
14	5.0	++++	316	6.3	150	47.4	99	31.3	16.1	3.2
15	11.0	++++	256	2.3	225	57.6	75	19.2	38.4	3.5
16	16	++++	719	4.5	150	107.85	45	32.35	75.5	4.7

\* Classification of shock:

++++ = death within 30 minutes;

+++ = marked fall in blood pressure with no recovery within 30 minutes,

++A = blood pressure at shock level for 20 minutes, but some recovery before 30 minutes;

\* ++B = blood pressure at shock level for 10 minutes, but with some recovery before 20 minutes;

++C = recovery before 10 minutes,

+ = mild shock with slight temporary fall in blood pressure;

0 = no recognizable shock symptoms.

† All histamine values in terms of histamine acid phosphate.

calculations of the liver histamine must be considered. It is well known that the dog's liver becomes markedly congested during anaphylactic shock and Weil (12) has estimated that at times as much as 60 per cent of the total circulating blood may be pooled in the liver. Most of this drains out freely upon removal of the liver. The increase in weight that may be due to edema,



etc., can only be estimated roughly by comparing the weight of the liver with that of normal dogs. The ratio of liver weight to body weight in dogs is stated by Welcker and Brandt (13) to be approximately 3.7 per cent. The liver weight in per cent of body weight in our experiments is indicated in the tables and it will be noted that there are only two experiments (Exps. 12 and 14) in which there is any evidence of a sufficient change in weight as to materially affect the computation. The results are shown in table 2.

#### DISCUSSION

It is quite apparent from the table that the amount of histamine lost by the liver during acute anaphylactic shock, when translated into terms of the dose in mgm. per kilo that this would represent, is roughly parallel to the degree of the shock experienced in each case. It is also possible to conclude from a study of the degree of effect produced by corresponding doses of histamine administered to normal dogs under comparable conditions (2) that the amounts of histamine liberated from the liver are substantially adequate to account for the degree of anaphylactic shock experienced. There are certain minor inconsistencies, but they are no more than can readily be attributed to either technical errors or normal variations in the susceptibility of animals. In addition to the observations of Waters and Markowitz, there is a great deal of evidence in the literature on anaphylaxis indicating that anaphylactic reactions are not the exclusive property of some one tissue. For example, although anaphylaxis in the guinea pig is predominantly a pulmonary affair, and it can readily be shown that perfusion of antigen through the isolated lungs of a sensitized guinea pig will produce a sufficient reaction of broncho-stenosis to account for the similar phenomenon in an intact animal, it has been repeatedly demonstrated that many other tissues from such a sensitized animal react upon contact with the antigen as the lungs do, and that they liberate histamine as a result of such contact (14). It is not surprising therefore that some anaphylactic manifestations can be induced in the dog even after hepatectomy. The evidence presented here, however, supports the large amount of earlier evidence which indicates that the liver has a dominant rôle in the acute anaphylactic reaction in the dog, and demonstrates more specifically, that the majority of the histamine which is liberated during the reaction comes from the liver.

#### CONCLUSION

Anaphylactic shock in dogs is accompanied by a reduction in liver histamine which is parallel to the severity of the reaction. The amount of histamine lost by the liver is substantially adequate to account for the severity of the shock in all cases so that it may be concluded that the liver plays the dominant rôle in this respect.



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# REDUCTION OF ARTERIAL BLOOD PRESSURE OF HYPERTENSIVE RATS BY ADMINISTRATION OF RENAL EXTRACTS

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Received for publication May 24, 1941

According to different investigators, the kidney not only can elaborate a pressor substance responsible for hypertension, but also a principle which will counteract the hypertension of renal origin. For recent articles dealing with the various aspects of experimental hypertension and its reduction, the reader is referred to the following references: (1-5).

In this communication we wish to report briefly our observations of the effect on the blood pressure of hypertensive rats of various extracts of kidney as well as certain other tissues.

## PREPARATION OF RENAL EXTRACTS

Twenty pounds of fresh frozen hog kidneys from which the fat had been removed were ground twice through a medium mesh plate and extracted for 4 hours under stirring with a 0.1 saturated solution of ammonium sulfate, using 1 liter per pound of kidney. The pH was adjusted to about 3.0 with 2 N HCl, and stirring continued for 2 hours. The extract was then filtered overnight through folded filter papers. The solid material was discarded, and to the clear filtrate solid ammonium sulfate was added to a concentration of 0.6 saturation. The solution was allowed to remain in the ice box overnight. The precipitate formed was collected on a Buchner funnel and suspended in distilled water, using from one to two liters per 20 pounds of kidney. The suspension was stirred thoroughly for 2 hours, the insoluble material removed by filtration, and ammonium sulfate added to the filtrate to a concentration of 0.2 saturation. After several hours, the precipitate formed was removed by centrifugation and was discarded. The supernatant liquid was again brought to 0.6 saturation with ammonium sulfate and kept in the ice box overnight. The resulting precipitate was removed by centrifugation and dissolved in about 500 cc. of distilled water, which was then brought to 0.25 saturation of ammonium sulfate. The resulting precipitate was centrifuged off and discarded and the supernatant liquid again brought to 0.6 saturation with ammonium sulfate and kept in the ice box overnight. This fraction, precipitated for the third time at 0.6 saturation from a 0.25 saturated solution, was removed by centrifugation, washed and dried with acetone and ether. The yield of this fraction, designated hereafter as *Fraction A*, ranged between 1 and 2 grams per kilogram of fresh kidney.

For further purification *Fraction A* was dissolved in water and dialyzed for 24 hours against distilled water which was changed once. Any insoluble material formed during dialysis was removed and discarded and the clear dialysate precipitated in 95 per cent acetone.<sup>1</sup> The resultant precipitate was collected, washed and dried with alcohol

<sup>1</sup> For complete precipitation it was found necessary to add a small amount of a saturated alcoholic solution of sodium acetate.



and ether (*Fraction B*). The yield was approximately 1 gram per kilogram of fresh kidney.

Additional purification was achieved by bringing the filtrate of the dialysate to 50 per cent acetone. The resulting precipitate, *Fraction C*, was centrifuged off, washed and dried with acetone and ether. The supernatant was brought to 95 per cent acetone concentration<sup>1</sup> and the resulting precipitate, *Fraction D*, containing most of the active material, collected and dried in the same way. The yields were approximately 0.6 gram for *Fraction C* and 0.5 gram for *Fraction D*, per kilogram of fresh kidney.

The same procedure was employed for making extracts of beef muscle and beef liver. Fractions corresponding to renal *Fraction B* were prepared by repeated precipitation at 0.6 saturation with ammonium sulfate and subsequent dialysis.

### ASSAY

To determine the activity of the extracts obtained by the procedure described above we used rats rendered hypertensive by subtotal nephrectomy (6) or by unilateral rayon wrapping and unilateral nephrectomy (7). We consider the latter method superior for the following reasons: (1) the operation can be performed in one step and has a lower mortality rate than subtotal nephrectomy. (2) Blood pressures exceeding 180 mm. Hg are obtained more frequently by this method. (3) Although we have, at necropsy, never found a fibrous hull about the kidney, we feel that the compression of the renal tissue by rayon wrapping, counteracting the compensatory hypertrophy of the kidney, produces the desired ischemia and resultant hypertension. It may be mentioned, however, that only a certain percentage of the surviving animals become hypertensive and that it may take from 2 to 6 months for hypertension to develop. The systolic blood pressures of the warmed, unanesthetized rats were determined by the plethysmographic method of Williams, Harrison and Grollman (8).

Rats were not used for assay until their blood pressure had established itself at a fairly constant level in excess of 150 mm. Hg. Extracts were usually administered intramuscularly twice daily for 4 days unless otherwise stated, and blood pressure readings were continued until the previous hypertensive level was re-established. We noted that after 4 assays the sensitivity of some rats to renal extracts decreased. At least 4 animals were used in the standardization of each preparation.

### RESULTS

The assays of various renal extracts in hypertensive rats are summarized in table 1 and figures 1 to 4. We also tested muscle and liver extracts and purified renin kindly supplied by Dr. Swingle.<sup>2</sup>

As can be seen from table 1 and figures 1 and 2, *Fractions A* and *B* from hog kidney produced a pronounced and sustained lowering of blood pressure in hypertensive rats when injected intramuscularly, although the potency seemed to vary with different batches. Our best preparation of *Fraction B* was potent at 100 mgm. total dose, corresponding to 58 grams of fresh kidney. However, any quantitative interpretation of the assays is rendered difficult on account of the variations in the blood pressure of hypertensive rats. A comparison

<sup>2</sup> According to Dr. Swingle 0.1 mgm. of the renin preparation per kilogram of body weight, given intravenously to anesthetized dogs over a period of two to five seconds, raised the mean arterial pressure between 30 and 40 mm. of mercury.



of the assays of Fractions B<sub>1</sub>, B<sub>2</sub> and B<sub>3</sub> seems to indicate that intramuscular administration was more effective than subcutaneous injection. Administration of Fractions B<sub>4</sub> and B<sub>5</sub> by stomach tube, even at much higher doses,

TABLE 1  
*Effect of tissue extracts on the blood pressure of hypertensive rats*

PREPARATION	TOTAL DOSE PER RAT	EQUIVA- LENT FRESH TISSUE	ADMINISTRATION			ANTI- PRESSOR EFFECT†
			Daily	Num- ber of days	Route*	
Renal fractions:		gm.	cc.			
A <sub>1</sub>	1 gram	90	1 2x	4	IM	++
A <sub>2</sub>	1 gram	100	1 2x	4	IM	++
A <sub>3</sub>	4 cc.	300	0.5 2x	4	IM	++
B <sub>1</sub>	250 mgm.	200	1 1x	4	IM	++
B <sub>2</sub>	200 mgm.	115	0.5 2x	4	IM	++
B <sub>2</sub>	100 mgm.	58	0.5 2x	4	IM	++
B <sub>3</sub>	300 mgm.	240	1 2x	4	Sub.	+
B <sub>3</sub> heat sol.			1 2x	4	Sub.	±
B <sub>3</sub> heat insol.			1 2x	3	Sub.	±
B <sub>4</sub>	1.4 gram	450	0.5 2x	4	St. T	±
B <sub>4</sub>	350 mgm.	112	0.5 2x	4	IM	++
B <sub>5</sub>	250 mgm.	150	0.5 2x	4	IM	++
B <sub>5</sub>	1 gram	600	0.5 2x	4	St. T	±
B <sub>5</sub>	750 mgm.	450		3	In food	—
C <sub>1</sub>	300 mgm.	300	0.75 2x	4	IM	±
D <sub>1</sub>	200 mgm.	250	0.5 2x	4	IM	++
D <sub>1</sub> heat sol.	150 mgm.		0.5 2x	4	IM	±
D <sub>1</sub> reheated	150 mgm.		0.5 2x	4	IM	±
D <sub>2</sub>	320 mgm.	260	0.5 2x	4	IM	++
D <sub>3</sub>	150 mgm.	225	0.5 2x	4	IM	++
D <sub>3</sub>	300 mgm.	450	0.5 2x	4	St. T	±
Muscle extract	4 cc.	300	0.75 2x	3	Sub.	±
Muscle extract	100 mgm.	400	0.5 2x	4	IM	±
Liver extract	4 cc.	450	0.5 2x	4	IM	—
Renin Swingle	70 mgm.		1 2x	4	IM	—

\* IM, intramuscular injection; Sub., subcutaneous injection; St. T., administration by stomach tube.

† ++, marked reduction, well sustained; +, marked reduction but not sustained; ±, some reduction; —, no effect.

proved to be much less efficacious than parenteral administration (fig. 3). Mixed with the food, Fraction B<sub>5</sub> failed to elicit any definite response. Fraction D, the 50 per cent acetone soluble material, was found in general to



possess high anti-hypertensive potency (fig. 4) while Fraction C, the 50 per cent acetone insoluble material, contained only traces of potency.

In normal rats, no lowering of blood pressure was observed upon administration of renal anti-hypertensive preparations.

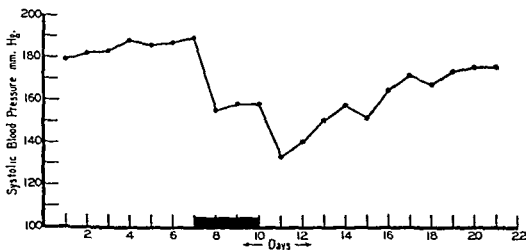


FIG. 1. EFFECT OF RENAL FRACTION A<sub>1</sub> ON BLOOD PRESSURE OF 5 HYPERTENSIVE RATS [INTRAMUSCULAR ADMINISTRATION]

The Curve represents the average daily readings. The block indicates the time of therapy. Total dose: 1 gram equivalent to 100 grams fresh kidney.

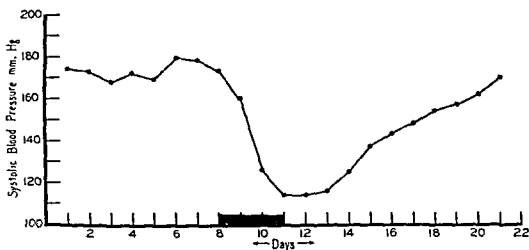


FIG. 2. EFFECT OF RENAL FRACTION B<sub>1</sub> ON BLOOD PRESSURE OF 10 HYPERTENSIVE RATS [INTRAMUSCULAR ADMINISTRATION]

The Curve represents the average daily readings. The block indicates the time of therapy. Total dose: 250 mgm. equivalent to 150 grams fresh kidney.

Fractions A and B, but not Fraction D caused a loss of body weight in the test animals when given parenterally. Deaths during the injection period were negligible, but some test rats failed to re-establish their previous hypertensive blood pressure levels after injection of Fractions A and B; such animals usually died within a week or 10 days after the assay.

Two extracts of beef muscle were found to produce only an insignificant



lowering of blood pressure. Liver extract and renin failed to influence the blood pressure of these hypertensive rats.

In acute experiments, different renal preparations were found to produce a pronounced pressor effect when injected intravenously into normal dogs and cats under Nembutal and Evipal anesthesia respectively. Obviously they were still contaminated with a pressor substance, probably renin. The

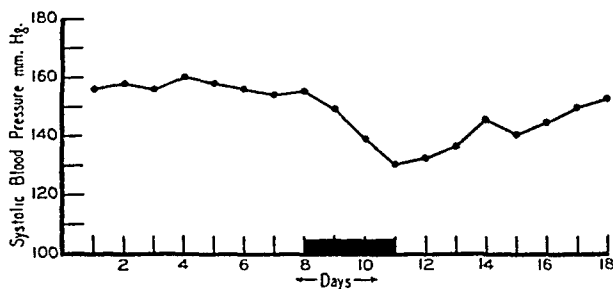


FIG. 3. EFFECT OF RENAL FRACTION B<sub>6</sub> ON BLOOD PRESSURE OF 4 HYPERTENSIVE RATS [ADMINISTRATION BY STOMACH TUBE]

The Curve represents the average daily readings. The block indicates the time of therapy. Total dose: 1 gram equivalent to 600 grams fresh kidney.

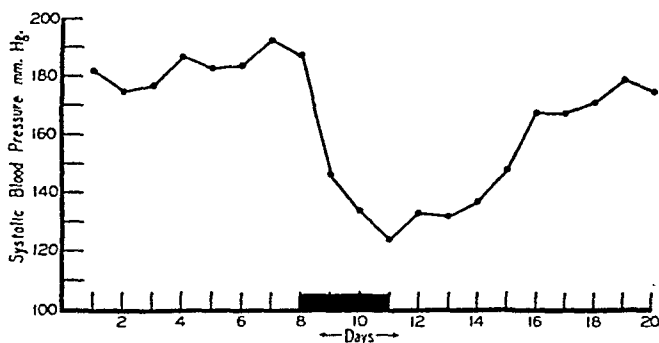


FIG. 4. EFFECT OF RENAL FRACTION D<sub>6</sub> ON BLOOD PRESSURE OF 6 HYPERTENSIVE RATS [INTRAMUSCULAR ADMINISTRATION]

The Curve represents the average daily readings. The block indicates the time of therapy. Total dose: 150 mgm. equivalent to 225 grams fresh kidney.

method of preparation of the renin is quite similar to the one employed by us for obtaining the anti-hypertensive factor from kidney (10). Preliminary attempts to separate the two factors have so far failed. Heating of the solution for 20 to 40 minutes at a temperature of 75°C. destroyed not only the pressor substance, but the anti-hypertensive factor as well as shown in the response of hypertensive rats (table 1).



## DISCUSSION

In general, our findings on the anti-hypertensive effect of various renal preparations in hypertensive rats are in agreement with similar observation reported by Grollman and his collaborators (11) and by Page and his co-workers (12). We found, however, that oral administration of renal preparations elicited only a slight response when given in four times the amount which upon intramuscular injection, gave a very pronounced and prolonged lowering in blood pressure.

Extracts of muscle and liver, prepared in a similar manner as renal extract, did not produce any definite anti-hypertensive effect in spite of the fact that they were given at a comparatively high dose level. It appears that the kidney is the best, if not the only tissue source for obtaining an anti-hypertensive preparation.

Our results are in agreement with the assumption expressed by different investigators (3-5) that the normal kidney elaborates a specific anti-hypertensive factor. At present practically nothing is known about the nature of this renal anti-pressor substance and about its mechanism of action. It might be mentioned in this connection that Schroeder and Adams (14) have recently reported that injection of tyrosinase preparations obtained from mushrooms lowers the blood pressure in hypertensive rats and dogs.

Another explanation of the anti-hypertensive effect of renal extracts arises from the observations of Wakerlin and his co-workers (15) that continuous administration of hog renin to hypertensive dogs produces a lowering of blood pressure while heat-inactivated hog renin and dog renin were ineffective. Since the serum of dogs and rabbits treated with renin was found capable of neutralizing the acute pressor effect of renin, an immune reaction to the heterologous hog renin seems to be responsible for the formation of "anti-renin." Although it is highly probable that the pressor substance present in our extracts is renin, it is doubtful that anti-hormone formation should take place in an acute experiment lasting only four days. Indeed, we found that injection of a purified hog renin preparation kindly supplied by Dr. Swingle failed to lower the blood pressure of hypertensive rats (table 1).

Further investigations will be necessary to determine the exact nature of the influence of the kidney on blood pressure.

## SUMMARY

1. A method is described for the preparation of renal extracts which are capable of reducing the blood pressure of rats with experimental hypertension. A total dose of 58 grams equivalent of fresh hog kidney, injected intramuscularly twice daily for a period of four days, was found to produce a pronounced and prolonged lowering of the blood pressure.

2. Oral administration of similar renal extracts at comparatively high doses failed to produce a comparable effect on the blood pressure.



3. Extracts of beef liver and muscle prepared according to the same procedure as employed for renal extracts, failed to lower the blood pressure in hypertensive rats.

4. The failure of hog renin to produce a lowering of blood pressure in hypertensive rats in our routine four day test seems to militate against the interpretation that the anti-pressor effect of renal extracts might be due to the formation of an anti-renin substance.

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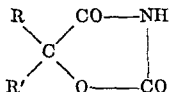
# HYPNOTIC ACTION OF 5,5-DIALKYL-2,4-OXAZOLIDINEDIONES WITH SPECIAL REFERENCE TO 5,5-DI-*n*-PROPYL-2,4- OXAZOLIDINEDIONE<sup>1</sup>

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Received for publication May 26, 1941

Recently, a series of new chemical compounds, the 5,5-disubstituted-2,4-oxazolidinediones, has been synthesized in this laboratory (1). These are closely related structurally to both the barbituric acids and the hydantoins.



Three different types have thus far been made. In the first of these, one *R* is always a methyl group while *R'* is some other alkyl group; in the second type, both *R* and *R'* are identical alkyl groups; and in the third, one *R* is always a phenyl group while *R'* consists of various alkyl groups. Most of these compounds are low-melting solids, very slightly soluble in water, but very soluble in oils and organic solvents. However, the hydrogen on the nitrogen is acidic and allows the formation of stable, water-soluble sodium and calcium salts.

The relative anesthetic properties and toxicity of the members of these three types of compounds were investigated by injecting aqueous solutions of the sodium salts into the tail veins of male albino mice. The median anesthetic doses (AD 50) of the different compounds were approximated by using five or more mice at each dose level on three or four points chosen as close as possible to the median anesthetic levels. The criterion of anesthesia was the inability of a mouse to right itself after pinching of the tail. The median lethal doses (LD 50) were also determined by intravenous injections in groups of five or more mice. The results are given in table 1.

The most active compounds were generally found to be those containing a total of eight to ten carbon atoms in the substituents of the 5-position. In this group, the anesthetic dose usually varied from 40 to 100 mgm. per kilo-

<sup>1</sup> The expense of this investigation was defrayed in part by a grant from the Mallinckrodt Chemical Works of St. Louis, Missouri.



gram, a range of activity approximately that of barbiturates. Three compounds, each of which contain highly branched alkyl chains (methyl tertiary-butyl, methyl neopentyl, and di-iso-propyl oxazolidinedione), showed definite hypnotic properties but also caused convulsions which were so violent that no attempt was made to determine the anesthetic dose. Two compounds (the phenyl and the diphenyl derivatives) showed no hypnotic action but the

TABLE 1  
*Anesthetic activity and toxicity of 5,5-dialkyl-2,4-oxazolidinediones*

2,4-OXAZOLIDINEDIONE	NUM- BER OF C ATOMS	M.P.	AD 50	LD 50	DURA- TION OF ANES- THESIA	REMARKS
		°C.	mgm./ kgm.	mgm./ kgm.	min.	
5- <i>n</i> -Butyl-5-methyl-.....	5	Oil	440	680	20.0	Convulsant
5- <i>tert</i> -Butyl-5-methyl-.....	5	85-86		750		
5- <i>n</i> -Amyl-5-methyl-.....	6	25	180	365	2.0	Convulsant
5-Neopentyl-5-methyl-.....	6	55-56		900		
5- <i>n</i> -Hexyl-5-methyl-.....	7	46-47	125	225	2.0	Recovery rapid
5- <i>n</i> -Heptyl-5-methyl-.....	8	32-33	95	145	1.5	
5-Methyl-5-(3-methylhexyl)-	8	Oil	85	155	2.0	Recovery rapid
5-Methyl-5- <i>n</i> -octyl-.....	9	62-63	70	125	2.0	Recovery rapid
5-Methyl-5- <i>n</i> -nonyl-.....	10	52-53	75	85	1.0	
5,5-Dimethyl-.....	2	76-77	400	450	1.0	Depressed 2-5 hours
5,5-Diethyl-.....	4	28	300	400	1.5	
5,5-Di- <i>n</i> -propyl-.....	6	42-43	172	315	15.0	Convulsant
5,5-Di- <i>iso</i> -propyl-.....	6	86-87		850		Recovery rapid
5,5-Di- <i>n</i> -butyl-.....	8	68-69	50	75	1.5	Some excitement
5,5-Di- <i>iso</i> -butyl-.....	8	Oil	95	120	2.0	Recovery rapid
5,5-Di- <i>n</i> -amyl-.....	10	63-64	40	70	3.0	
5-Phenyl-.....	6	107-108	725	725		Convulsant
5-Methyl-5-phenyl-.....	7	73-74	550	575	300.0	
5-Ethyl-5-phenyl-.....	8	61-62	200	300	25.0	Depressed 1-2 hours
5-Phenyl-5- <i>n</i> -propyl-.....	9	Oil	140	205	5.0	
5- <i>n</i> -Butyl-5-phenyl-.....	10	63-64	90	115	2.5	Recovery rapid
5- <i>iso</i> -Butyl-5-phenyl-.....	10	Oil	115	145	3.0	Depressed 20-30 min- utes
5- <i>n</i> -Amyl-5-phenyl-.....	11	Oil	65	90	2.0	Recovery rapid
5,5-Diphenyl-.....	12	135-136	135	135		Convulsant

animals died in convulsions. The remaining compounds produced anesthesia with little or no excitement. Anesthesia came on almost at once after the intravenous injection of an anesthetic dose and was of relatively short duration. The values given for the average duration of anesthesia are those obtained from a dose midway between the anesthetic and lethal doses. Recovery was usually very rapid, but with a few compounds a certain amount



of hypnosis or sedation was noted in the animals after the initial anesthesia had passed off. In the case of the di-*n*-propyloxazolidinedione this hypnosis was of long duration and of such a character that further investigation of this particular derivative was undertaken. The other members of the series are to be investigated later.

#### ACTION OF 5,5-DI-*n*-PROPYL-2,4-OXAZOLIDINEDIONE

*Relationship of anesthetic activity to toxicity.* The anesthetic and the lethal doses of di-*n*-propyloxazolidinedione were determined more exactly than in the preliminary experiments by using a larger number of mice. The results are summarized in table 2. The median anesthetic dose was found to be  $172 \pm 5$  mgm. per kilogram and the median lethal dose to be  $315 \pm 9$  mgm. per

TABLE 2

*Effect of intravenous administration of di-*n*-propyloxazolidinedione as the sodium salt to white mice*

DOSE mgm./kgm.	NUMBER OF MICE	PER CENT ANESTHETIZED	PER CENT DEAD
160	16	31.2	0
170	15	46.6	0
175	15	53.3	0
180	15	60.0	0
190	12	91.7	0.
250	4	100.0	0
300	15	100.0	40.0
310	15	100.0	46.7
320	15	100.0	53.3
330	15	100.0	60.0

AD 50 =  $172 \pm 5$  mgm./kgm.; LD 50 =  $315 \pm 9$  mgm./kgm.

kilogram. These doses and their standard errors were estimated by the method of Bliss (2).

*Response to intravenous doses of different sizes.* A five or ten per cent solution of di-*n*-propyloxazolidinedione as the calcium or sodium salt was used in these experiments. The calcium salt was most generally given, although later the sodium salt was used in its place to avoid the introduction of large amounts of calcium. There seemed to be no appreciable difference in hypnotic effect on animals between these two salts.

Fifteen dogs were given intravenously a total of 70 doses ranging in size from 25 to 350 mgm. per kilogram. Doses of 25 to 50 mgm. per kilogram caused muscular incoordination. The dogs became unsteady on their feet but apparently felt quite well and took food readily. However, they would sleep quietly for several hours if they were not disturbed. Doses of 125-175 mgm. per kilogram caused a period of light anesthesia during which the



animals were quiet and relaxed but gave some response to painful stimuli. The onset was immediate and without excitement. This period of anesthesia lasted for approximately half an hour, but for the next 24 hours they were unable to stand alone. They remained in a state of relaxation, sleeping most of the time with occasional periods of whining and movements of the extremities, followed again by sleep. During the following 24 hours they slept quietly if undisturbed but could be aroused and given food and water. Doses of 200 mgm. per kilogram produced deep anesthesia for one half to one hour, which was followed by a period of lighter anesthesia lasting as long as 60 hours, the average being about 40 hours. For 24 hours following this the animals slept if undisturbed. Doses of 250 mgm. per kilogram produced a very deep anesthesia during which the wink reflex and all responses to stimuli were commonly lost. These dogs were unable to stand for an average of 42 hours after the injection and would sleep even longer if undisturbed. Death from such a dose was very rare. Doses larger than 250 mgm. per kilogram, which were only rarely used, sometimes caused the death of the animal. Surprisingly few after-effects of the drug were evident when the dogs were once able to stand and walk about. They seemed to feel well, and showed an interest in their surroundings and in their food.

*Response to oral doses.* The response of dogs to oral doses of various sizes was similar to that obtained on intravenous administration except that there was a lag of 15 to 30 minutes before onset of action of the drug; the effects persisted for the same length of time. Administration of 25 mgm. per kilogram, the smallest dose that had a readily noticeable effect, produced slight muscular incoordination. If not disturbed the animals slept for 24 hours or longer but could be aroused at any time to take food and water. By increasing the dose an increasingly deep sleep was obtained until, at doses of 300 mgm. per kilogram, animals would not react to painful stimuli for a period of 24 hours and would sleep for 48 hours longer. It was observed that the drug was also effective after rectal administration to dogs.

*Respiration.* This was recorded by means of a Palmer pneumograph connected to a Becker respiratory tambour. There was little change in the respiration when the drug was injected intravenously. A slight diminution in the depth of respiration without much change in rate was noted when a sufficient amount of the drug was injected to produce anesthesia. The depth then gradually increased and the rate decreased somewhat. Respiration was entirely adequate except after very large doses of above 250 mgm. per kilogram.

*Cardiac rate.* There was a uniform increase in heart rate following the administration of dipropylloxazolidinedione in dogs similar to that observed after barbiturates. The initial rate in most of the animals used was between 80 and 100 and this increased to between 150 and 250 following injection of the drug. The maximum rate was usually reached soon after injection, but remained above the control rate as long as the effect of the drug persisted.



*Blood pressure.* The femoral artery was cannulated under procaine anesthesia and when the drug was slowly injected intravenously even to the point of complete anesthesia there was no appreciable effect on the blood pressure. If injected more rapidly, (3 to 5 cc. of the 10 per cent solution per minute), there was a temporary fall in blood pressure from between 10 to 20 mm. with prompt return to normal. In some instances the blood pressure remained about 10 mm. below normal during the experiment but more often rose to normal or above, 30 to 35 minutes after injection. In no instance was a serious fall in blood pressure observed.

*Electrocardiogram.* A standard Cambridge electrocardiograph was used. All records were from lead II. Records were taken before and at intervals during the different stages of anesthesia in each dog. They uniformly showed an increase in heart rate as previously described following the injection of the drug. No arrhythmias or change in the P-R interval or in the form of the electrocardiographic curve that could be interpreted as resulting from a toxic effect of the drug were encountered.

*Blood sugar.* Blood sugar determinations were made before (fasting levels) and one hour after the administration of the drug using the Folin-Wu method. No consistent change in blood sugar level was observed.

*The effect of chronic administration.* Eight dogs were used, all of which were observed for a week before treatment began and given a thorough physical examination, including blood and urine examination. The dogs were given 8 to 10 doses intravenously, varying in size from 25 to 300 mgm. per kilogram at intervals of one to two weeks. The animals were then observed for 3 months and the injections repeated at the same intervals and in the same number. Two of these dogs died after administration of the drug. One vomited and aspirated pieces of meat and bone were found in the bronchi at autopsy; the other died after prolonged anesthesia following a dose of 250 mgm. per kilogram when sufficient care was not taken to keep the animal warm while under the influence of the drug.

The urine was examined at intervals for sugar, albumin and microscopically for cellular changes, but no abnormalities were found.

During the course of treatment red cell counts, white cell counts, hemoglobin determinations and differential counts were made several times on each dog. No significant changes were observed. In one dog the blood gases, the blood pH, and the icteric index were followed after the administration of the drug and showed no abnormal changes. Another dog delivered four full term pups a few days after recovering from 250 mgm. per kgm. dose of the drug.

Five of the dogs which received the two courses of treatment were killed while under the influence of the final dose and autopsies were performed. Complete gross examinations and microscopic examinations of sections of the liver, kidneys, adrenals, pancreas, heart and spleen were made. No changes that were considered significant were found. Especial attention was paid to the livers of these animals. In one there was a certain amount of fatty



infiltration, but as the same picture is found in normal dogs under similar conditions, and as the other treated dogs showed no fatty infiltration, it was felt this was of no significance.

Recently, this substance has been given to a selected group of patients both intravenously and by mouth and they have shown essentially the same picture as seen in the animals described here. A report on this work is being published elsewhere (3).

#### SUMMARY

A series of new chemical compounds, the 5,5-dialkyl-2,4-oxazolidinediones, has been presented which exhibits hypnotic action. Their relative anesthetic activity in white mice has been evaluated. The anesthesia produced was usually of brief duration with rapid recovery.

5,5-Di-*n*-propyl-2,4-oxazolidinedione has been given intravenously, orally and by rectum to a series of animals in varying doses over different periods of time. All degrees of anesthesia and hypnosis have been produced by this substance. On the intravenous administration of full anesthetic doses to dogs, the period of anesthesia was relatively short, but was followed by a period of hypnosis which might last for 60 or 70 hours. A careful study of the reaction of the animals to this substance has been made and no contraindications to its trial in man have thus far been discovered. It has been given to a small series of patients without ill-effects and appears to deserve further clinical trial.

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# THE DIFFERENTIAL EXCRETION OF BROMIDE AND CHLORIDE IONS AND ITS RÔLE IN BROMIDE RETENTION<sup>1</sup>

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Received for publication May 27, 1941

The problem of bromide retention has assumed renewed importance within recent years (1). It has been shown within the past decade that the incidence of bromide intoxication is much greater than it was formerly thought to be (2, 3, 4, 5). Attention has been redirected to the association of this intoxication with mental disturbances, and the wide and subtle variety of these disturbances has been the object of considerable study (4, 6).

It has long been appreciated that the excretions of bromide and chloride are interdependent (7, 8, 6). It has been shown that the excretion of bromide or, conversely, its retention is related to the bromide: chloride ratio in the food (9). The degree of bromide retention is quite obviously also dependent upon the ratio in which the chloride and bromide ions are excreted by the kidney. Though this excretion has been investigated by several workers (10-21) considerable difference of opinion still exists as to whether the kidney differentiates between these two ions, and, if it does, as to the consequent rôle in bromide retention (22, 23).

We have gathered data concerning these questions by following in detail: (a) the fate of single intravenous doses of sodium bromide injected into fasting dogs which were excreting basal, minimal amounts of chloride; (b) the effect of single intravenous injections of sodium chloride into dogs containing bromide in their tissues; (c) the effects of salyrgan, theophylline, and sodium sulfate administered to dogs which had previously been injected with bromide. The relation of these data to other factors involved in bromide retention is also discussed. In the present experiments we have assumed, in agreement with reports found in the literature, that the chloride and bromide ions are excreted practically entirely through the kidney (24, 25, 26, 14).

## EXPERIMENTAL

Healthy female dogs were kept without food but were given water for the duration of the experiment, usually 2 to 3 weeks. Four series of experiments involving 3 animals were carried out. After a preliminary period of fasting, the plasma chloride concentra-

<sup>1</sup> This research was aided by a grant from the Emulsion Drug Company to Cornell University Medical College.



tion and the excretion of chloride were determined; the latter amounted to about 0.1 mM per hour. Measured amounts of sodium bromide or sodium chloride were injected intravenously and the subsequent excretion of bromide and chloride and the concentrations of these ions in the plasma were determined at 1 hour after injection, 3 hours after injection, and thereafter, at approximately 24-hour intervals. The influence of various diuretics was also studied as described subsequently. In most of the experiments the volumes of ingested water and of urine were measured.

The urinary chloride was determined by the modified Volhard-Harvey titration (27), the plasma chloride or halide, by means of the open Carius method as given by Peters and Van Slyke (28). The blood was collected and centrifuged under oil. Urinary bromide was determined by the method of Brodie and Friedman (29); the accuracy of this method has been discussed by these investigators. For determination of plasma bromide, it was found convenient and satisfactory to use a method depending on the deep orange yellow color produced by the interaction of the bromide ion and gold chloride, carried out in the trichloroacetic acid filtrate. This method has been used by many investigators (30, 6). The color intensity was measured photoelectrically with a light blue green pyrex glass filter (no. 428). Recovery experiments were carried out to gain assurance that the color intensity was indeed a measure of the bromide concentration. In our hands duplicate or triplicate values usually checked within 2 to 3 per cent.

All quantities of halides have been expressed in molecular terms. For our present purposes it was considered adequate to express the concentration in the plasma as millimols per liter of plasma, and not to correct to millimols per liter or kilogram of serum or plasma water. The following terms have been calculated in accordance with usage present in the literature: the urinary bromide:halide ratio, obtained by dividing the number of millimols bromide excreted ( $Br_u$ ) during a given period by the number of millimols of total halide (bromide plus chloride) excreted ( $H_u$ ) during the same period; the plasma bromide:halide ratio obtained by dividing the average concentration of plasma bromide ( $Br_p$ ) during the period of excretion by the average concentration of plasma halide ( $H_p$ ) during this period. These ratios when multiplied by 100 indicate the molecular percentage of halide which has been replaced by bromide.

If the percentage of bromide in the urinary halide is equal to that in the plasma halide, then it follows that the kidney is treating the bromide and chloride ions in the same manner, most probably by the tubules reabsorbing these ions to the same extent from the glomerular filtrate. If, on the other hand, the percentage of bromide in the urinary halide is less than in the plasma halide, it follows that bromide is being excreted less readily than chloride, most probably by the tubules reabsorbing the bromide ions to a greater extent than the chloride ions. The ratio of these percentages:  $\frac{Br_u/H_u}{Br_p/H_p}$  is thus an expression of the degree to which bromide is excreted less readily, or conversely, of the degree to which chloride is being excreted preferentially. In accordance with usage in the literature, the ratio of these percentages has been designated as  $K$  and will be so referred to in the course of this paper.

## RESULTS

### *Excretion of bromide after a single intravenous injection of bromide*

The effects of injecting single intravenous doses of sodium bromide into fasting animals excreting basal, minimal amounts of chloride are shown in table 1. A considerable excretion of bromide resulted within the first hour; after this period the amount excreted per hour decreased, though it was still



considerable for approximately the next 20 hours. Thus, in dog 1, after an injection of 4.85 mM per kilogram or a total of 80.5 mM, 0.45 mM was excreted during the first hour. During the next 2 hours, 0.36 mM or 0.18 mM per hour was excreted, and during the subsequent 21 hours, 0.87 mM or an average of 0.041 mM per hour was excreted. When larger amounts of

TABLE 1  
*Effect of bromide injection on halide excretion*

PERIOD	DURATION	WATER INGESTED	URINE				BLOOD PLASMA			K
			Volume	Halide	Bromide	Bromide Halide	Halide	Bromide	Bromide Halide	
Dog 1. 4.85 mM/kgm. of bromide injected; total of 80.5 mM.										
	hours	cc./hour	cc./hour	mM/ hour	mM/ hour		mM/l.	mM/l.		
3/20	0						106	0.0		
3/20	1			6.62	.45	.068	109	16.1	.148	.46
3/20	2			3.44	.18	.052	108	16.4	.152	.34
3/20-3/21	21			0.96	.041	.043	109	16.2	.148	.29
3/21-3/22	23.8			0.36	.012	.033				
3/22-3/23	24.3			0.30	.010	.032	109	16.6	.152	.21
Dog 2. 9.70 mM/kgm. of bromide, total of 83.4 mM.										
4/11	0	6	6				101	0.0		
4/11	1	0	35.0	15.9	3.85	.242	111	28.4	.245	.99
4/11	2	0	15.0	4.68	.84	.168	110	29.7	.270	.62
4/12	20	12.5	7.2	1.28	.16	.125	98	28.7	.290	.43
4/13	24	4.2	2.9	0.062	.075	.120	102	27.5	.270	.44
Dog 1a. 7.28 mM/kgm. of bromide; total of 109.3 mM.										
5/9	0	5.2	3.3				108	0.0		
5/9	1	0	60	19.7	3.09	.157	120	22.8	.190	.83
5/9	2	0	60	18.6	2.62	.141	116	22.3	.193	.73
5/10	21	12	6.4	1.87	.23	.123	111	21.6	.196	.63
5/11	24	0	2.9	0.28	.027	.096	111	21.2	.192	.50
Dog 3. 7.28 mM/kgm. of bromide; total of 65.0 mM.										
6/6	0						107	0		
6/6	1.16	0	50	5.08	.57	.112	117	20.5	.175	.64
6/6	1.5	0	20	4.90	.46	.094	116	20.8	.179	.52
6/6	21	12.4	4.8	1.26	.12	.096	108	20.4	.190	.50

sodium bromide were injected as, for example, 9.70 mM per kilogram in dog 2, the amounts of sodium bromide excreted were considerably larger.

As is well known and as has already been mentioned, excretion of bromide is associated with an excretion of chloride. A study of the relation between the amounts of bromide and chloride excreted during various periods after



injection, as listed in table 1, shows that the bromide:halide ratio was highest during the first hour after injection, and decreased thereafter. Thus, in dog 1 the urinary bromide:halide ratio was 0.068 during the first hour, 0.052 during the next 2 hours, and fell to a value of about 0.032 for the second and third days after injection. Again in dog 2, following the injection of a larger amount of sodium bromide (9.70 mM per kilogram) the ratio was 0.242 during the first hour, 0.168 during the next 2 hours, and 0.125 during the following 20 hours. The ratios in dogs 1a and 3 followed a similar course.

When the urinary bromide:halide ratios are compared with the plasma bromide:halide ratios for corresponding periods, it is seen that  $K$  or  $\left(\frac{Br_u/H_u}{Br_p/H_p}\right)$  was highest during the first hour after injection. Thus, in dog 1  $K$  had a value of 0.46 for the first hour after injection, 0.34 for the next 2 hours, and 0.29 for the subsequent 21 hours. In dog 2, when a larger amount of sodium bromide was injected  $K$  had a value of 0.99 for the first hour, 0.62 for the next 2 hours, and 0.43 for the next 20 hours. These relatively higher values of  $K$  immediately following the injections indicate that during these periods the kidney differentiates less markedly between the bromide and chloride ions than it does later. In the case of dog 2, where the value of  $K$  was 0.99 for the first hour, there was no differentiation between these two ions.

About 24 to 48 hours after the injection of sodium bromide the excretion of chloride and bromide became negligible. When the total amount of halides excreted up to that time, or even up to 3 or 4 days, was calculated, it was found that the result was not necessarily equivalent to the amount of bromide that had been injected. Closer scrutiny revealed that the amount excreted depended to a considerable extent on the state of halide saturation of the animal. Thus, dog 1 had been fasted for 9 days before the injection of bromide. During the 24 hours following the injection of 80.5 mM of sodium bromide, 33.7 mM of halide were excreted; this represented an excretion of 47 per cent of the injected halide. Dogs 2 and 1a were fasted for only 5 days; the excretions were relatively greater, i.e. 60 and 88 per cent of the injected doses, respectively. This failure to excrete a molecularly equivalent amount of halide is due, as will be demonstrated, to retention because of previous halide depletion.

#### *Excretion of bromide after a single intravenous injection of sodium chloride*

As has already been stated, about 2 to 3 days after the injection of sodium bromide, the excretion of halide became negligible. In spite of the presence of high concentrations of bromide in the plasma, very little or no bromide was being excreted. A single intravenous injection of sodium chloride at such a stage led to a very prompt and marked excretion of bromide. Thus, in table 2 it may be seen that the injection of 4.27 mM per kilogram of sodium chloride in dog 1 led to an excretion of 0.35 mM of bromide within the first hour after



injection, to 0.83 mM or 0.41 mM per hour during the next 2 hours, and to 0.92 mM or 0.044 mM per hour during the subsequent 21 hours. The other data listed in table 2 illustrate the same effect.

As was to be expected, the excretion of bromide was associated with that of chloride. The bromide:halide ratio in the urine tended to be slightly higher immediately after the injection than later. These ratios were not so high as those obtained after the bromide injections, nor did they decrease as markedly with time. The values of  $K$  also reflected these findings. Thus, in the case of dog 2 where 8.54 mM of sodium chloride was injected,  $K$  had a

TABLE 2  
*Effect of chloride injection on halide excretion*

PERIOD	DURATION	WATER INGESTED	URINE				BLOOD PLASMA			K
			Volume	Halide	Bromide	Bromide Halide	Halide	Bromide	Bromide Halide	
Dog 1. 4.27 mM/kgm. of NaCl injected; total of 70.9 mM.										
	hours	cc./hour	cc./hour	mM/hour	mM/hour		mM/l.	mM/l.		
	0						111	16.5	.149	
3/27	1			6.92	.35	.050	121	16.0	.132	.38
3/27	2			5.88	.41	.070	115	16.2	.141	.50
3/28	21			0.68	.044	.064	111	15.1	.136	.47
3/29	24			0.24	.013	.052			(.136)	.38
Dog 2. 8.54 mM/kgm. of NaCl injected; total of 74.0 mM; after salyrgan diuresis										
4/18	0	8.3	5.8				89	24.6	.277	
	1	0	30	5.69	.90	.158	107	22.4	.210	.75
	2	0	15	3.12	.48	.154	106	21.6	.203	.76
4/19	21	10.6	4.6	0.28	.045	.159	103	21.2	.206	.76
Dog 1a. 8.54 mM/kgm. of NaCl injected; total of 128.9 mM.										
5/11	0	0	2.9				111	21.2	.190	
	1	0	110	31.3	3.83	.123	126	20.8	.166	.74
	2	0	78	25.3	2.98	.118	121	19.7	.162	.73
5/13	45	0	5.1	1.73	.19	.108	110	21.5	.180	.60

value of 0.75 during the first hour after injection, and practically the same value during the next period of 2 hours, and the subsequent one of 21 hours.

In these instances also it was noted that the amount of total halide excreted during a given period depended upon the state of halide saturation of the animal. For example, as may be seen in table 3-b, 70.9 mM of chloride were injected into dog 1 seven days after a previous injection of bromide; 33.1 mM of halide as chloride and bromide were excreted within the next 24 hours. This represented a 47 per cent excretion. In dog 2, 74.0 mM of sodium chloride were injected after halide depletion resulting from a diuresis induced



by salyrgan; in 24 hours, 17.9 mM of halide, or only 24 per cent of the injected dose were excreted. On the other hand, 2 days after the injection of this amount of sodium chloride, when the animal was obviously more saturated with respect to halide, another injection of 29.8 mM of sodium chloride led to an excretion of 26.6 mM of halide, or 89 per cent of the injected dose, in 24 hours.

*Relation of bromide excretion to the concentrations of bromide and chloride in the plasma*

The presence of bromide in the blood and in the tissues does not necessarily imply that this ion will be excreted. As has just been mentioned, about 2 to

TABLE 3  
*Excretion of bromide and chloride in 24-hour period*

DOG	$H_i$	$H_u$	$Br_u$	$\frac{H_u}{H_i}$	$\frac{Br_u}{H_i}$	$\frac{Br_u}{H_u}$	$\frac{Br_p}{H_p}$	$\frac{Br_p}{H_p} \cdot \frac{H_u}{H_i}$
	HALIDE INJECTED	HALIDE EXCRETED	BROMIDE EXCRETED	HALIDE EXCRETED HALIDE INJECTED	BROMIDE EXCRETED HALIDE INJECTED	URINARY BROMIDE HALIDE	PLASMA BROMIDE HALIDE	
a. Following injection of sodium bromide								
	mM	mM	mM	per cent	per cent			
1	80.5	33.7	1.68	42	2.1	.050	.15	.063
2	83.4	50.8	8.72	60	10.4	.172	.27	.162
1a	109	96.0	13.2	88	12.1	.137	.19	.167
3	65.0	39.6	3.87	61	5.9	.098	.18	.110
b. Following injection of sodium chloride								
	mM	mM	mM	per cent	per cent			
1	70.9	33.1	2.10	47	3.0	.064	.14	.066
2	74.0	17.9	2.81	24	4.0	.159	.22	.053
2	29.8	26.6	3.40	89	11.4	.127	.20	.178
2	59.6	63.4	7.39	106	12.4	.117	.17	.180
1a*	128.9	159.8	18.2	126	14.1	.114	.17	.214

\* 48-hour period.

3 days after an injection of sodium bromide the excretion of both bromide and chloride became negligible. Yet at this stage the concentration of plasma bromide ranged, in the various experiments performed, from 16.6 to 28.7 mM per liter of plasma. Whether bromide was excreted in more than negligible amounts depended not on the presence of bromide in plasma and tissues, but rather upon the sum of the concentrations of both bromide and chloride in the plasma, i.e., upon the concentration of total halide in the plasma. This may be briefly illustrated by figure 1. After several days of fasting, dog 1a showed a plasma chloride concentration of 108 mM per liter and an hourly excretion of 0.16 mM of chloride. After the injection of 7.28



mM per kilogram of sodium bromide, the concentration of halide in the plasma rose to 120 mM per liter; the excretion of bromide during the first hour was 3.09 mM. By the end of the third hour the plasma concentration was 116 mM; the hourly excretion of bromide during the intervening 2 hours had fallen to 2.62 mM. At the end of another 21 hours the plasma concentration of halide decreased to 111 mM per liter; the average hourly excretion during these 21 hours had been 0.23 mM of bromide. By the end of 48 hours after the injection the excretion of bromide became negligible,—0.03 mM per hour.

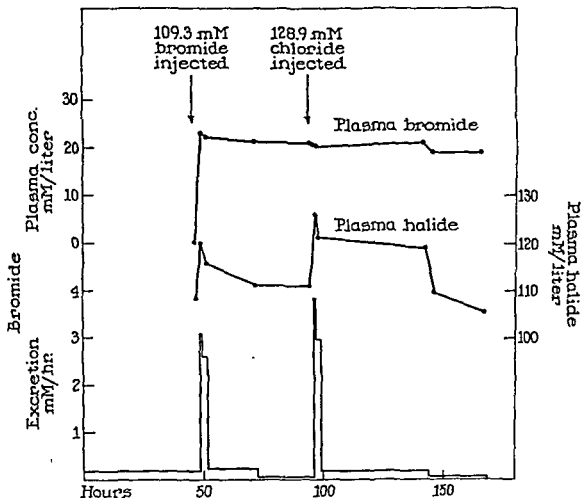


FIG. 1. RELATION OF BROMIDE EXCRETION TO THE CONCENTRATION OF BROMIDE AND TOTAL HALIDE IN THE PLASMA. Dog 1a

Though at this stage the concentration of plasma bromide was still almost as high as when excretion first began, the plasma halide concentration was at a low level—110 mM per liter. The excretion of bromide was again initiated when the concentration of total halide was increased, even though this was accomplished through the injection of sodium chloride and even though the concentration of plasma bromide fell slightly. In short, as may be seen from Figure 1, bromide excretion parallels the curve of the concentration of total halide in the plasma, and not that of the bromide concentration.



We have already noted, as have previous workers, the relation between urinary and plasma bromide:halide ratios:

$$\frac{Br_u/H_u}{Br_p/H_p} = K \dots\dots\dots (1)$$

We have also observed that  $K$  had different values, depending upon the circumstances under which bromide excretion proceeded. Thus, after the injections of large amounts of sodium bromide (table 1), the value of  $K$  was high, 0.9 to 1.0, and decreased rather rapidly within 24 hours to about 0.4 to 0.5. After injections of large amounts of sodium chloride the value of  $K$  was about 0.7 and did not decrease so rapidly. The injection of smaller amounts of sodium bromide or chloride led to smaller values of  $K$ , about 0.4 to 0.5. When, however, the urinary and plasma bromide:halide ratios were determined for the first 24 hours after injection as a unit, it was found that the value of  $K$  was usually about 0.65. This value was obtained whether the excretion followed the injection of bromide or of chloride. It was obtained following the injection of a small amount of halide, 3.42 mM per kilogram (dog 2, table 3-b) as well as following the injections of the larger amounts. The only exceptions occurred when relatively small amounts of bromide (4.85 mM per kilogram) and of chloride (4.27 mM per kilogram) were injected into dog 1 which was considerably halide-depleted at these times; in these instances,  $K$  was about 0.4.

Equation 1 may be transposed to express the amount of bromide excreted during a given period as a fraction of the amount of halide injected ( $H_i$ ) at the beginning of the period.

$$\frac{Br_u}{H_i} = K \cdot \frac{Br_p}{H_p} \cdot \frac{H_u}{H_i} \dots\dots\dots (2)$$

It may thus be seen that the amount of bromide excreted depends upon  $H_i$ , the amount of halide injected;  $Br_p/H_p$ , the plasma bromide:halide ratio;  $H_u/H_i$ , the state of halide saturation of the tissues, and finally, upon the value of  $K$ . We have seen that, for a 24-hour period following injection and under the conditions described above,  $K$  has a value of about 0.65. In figure 2 are plotted the amounts of bromide excreted, as fractions of injected halide, against the plasma bromide:halide ratios multiplied by the state of saturation of the tissues. It may be seen that most of these points fall on a straight line which has a slope,  $K$ , equal to about 0.65, thus bearing out the relationship expressed by Equation 2.

#### EFFECTS OF DIURETICS

In tables 1 and 2 is shown the diuresis produced by the injection of sodium bromide or sodium chloride. Thus in dog 2, during the 24 hours preceding the injection of 83.4 mM of sodium bromide the average urine output was



6.0 cc. per hour. During the first hour after injection the output rose to 35.0 cc. per hour and averaged 15.0 cc. for the next 2 hours. In general, during these periods of diuresis, as has already been pointed out, the ratio of bromide to halide in the urine was higher than in subsequent periods when there was no diuresis.

The administration of large amounts of water by stomach tube in one animal (dog 1a) did not appreciably increase the excretion of halide although the

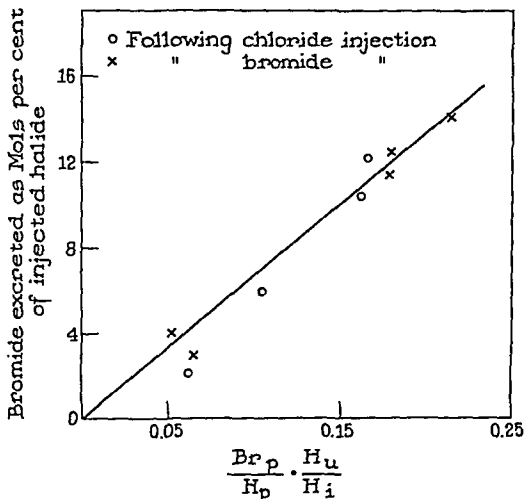


FIG. 2. RELATION OF THE AMOUNT OF BROMIDE EXCRETED IN 24 HOURS TO THE PLASMA BROMIDE:HALIDE RATIO,  $\frac{Br_p}{H_p}$ , AND THE STATE OF HALIDE SATURATION OF THE ANIMAL,  $\frac{H_u}{H_i}$ .

urine output was increased. Thus, on one occasion the urine output averaged about 4 to 5 cc. per hour, and the halide excretion 0.14 mM per hour. 1250 cc. of water was given by stomach tube and the animal drank another 150 cc. The average urine output during 20 hours following the administration of the water rose to 11.8 cc. per hour and the halide excretion averaged about 0.03 mM per hour. The plasma bromide was 18.5 mM per liter and did not change during this period.



*Effect of sodium sulfate*

0.4 gram of sodium sulfate per kilogram was injected intravenously into dog 1a. During the next  $2\frac{1}{2}$  hours there was a marked diuresis (an average of 102 cc. per hour). This diuresis was not accompanied by an appreciable increase in the excretion of either total halide or bromide. In another animal (dog 3) a similar dose of sodium sulfate produced an average diuresis of 44 cc. per hour during  $2\frac{1}{2}$  hours following the injection. The average excretion of halide amounted during this period to 0.2 mM per hour. During the following 22 hours the water excretion was 9.0 cc. per hour and the average halide excretion was 0.02 mM per hour. The excretions of bromide were correspondingly low. Because of these very low excretions of halide the percentage errors in the determination were high and it was not considered significant to calculate the bromide:halide ratios in the urine.

Theophylline increased the excretion of total halide and of bromide as well as the volume of urine. In dog 1a, 50 mgm. per kilogram of theophylline were injected intravenously. The urine flow, which had averaged about 10 to 11 cc., rose to 80 cc. per hour during the first hour, 50 cc. per hour during the next 2 hours, and 18 cc. per hour during the next 22 hours. The total halide excretion, which had averaged between 0.01 to 0.03 mM per hour preceding the injection, rose sharply to 4.74 mM during the first hour following the injection and averaged 7.64 mM per hour during the next 2 hours. The corresponding bromide excretions during these periods of diuresis were 0.83 and 1.34 mM per hour. The bromide:halide ratio in the urine during these two periods was 0.175. The corresponding ratios in the plasma were 0.181 during the first hour following injection and 0.174 in the subsequent 2 hours. Previous to the theophylline injection, the urinary bromide:halide ratio was 0.108, the plasma ratio, 0.180, and  $K$  was 0.6.  $K$  was 0.96 during the first hour after ingestion and 1.0 during the next 2 hours.

In dog 3 the excretion of halide during the 48 hours preceding the injection of the same dose of theophylline sodium acetate averaged 0.183 mM per hour. The excretion of bromide averaged 0.0134 mM per hour. The bromide:halide ratio in the urine was 0.073, whereas in the plasma it was 0.192 and  $K$  was therefore 0.38. After the injection of theophylline the total halide excretion rose to 2.28 mM during the first hour; bromide excretion rose to 0.24 mM. The bromide:halide ratio was 0.105, whereas that in the plasma remained at about 0.19, and  $K$  therefore rose to 0.55. During the next 2 hours the hourly excretion averaged 1.26 mM of halide, 0.151 mM of bromide; the bromide:halide ratio in the urine was 0.12, and  $K$  was 0.63. During these 3 hours the excretion of water amounted to about 3 cc. per hour. In this animal, therefore, although the effect of theophylline was not as marked as in dog 1a, there was nevertheless an increase in the total halide and bromide excretion, and an increase in the value of  $K$ .



*Effect of salyrgan*

Salyrgan was injected intravenously in dogs 1 and 2. The dose in each case was 5 mgm. per kilogram. The injection led to an enormous increase in the excretion of total halide consisting of increases in both chloride and bromide and to a rise in the bromide:halide ratio of the urine. These increases were accompanied by decreases in the plasma halide, which in turn consisted of decreases of both the chloride and the bromide. The effect is illustrated in table 4. It may be noted that on the third day preceding the injection of salyrgan the total halide excretion was 0.067 and the bromide excretion was 0.0073 mM per hour. The urinary bromide:halide ratio was 0.109; the plasma bromide:halide ratio was 0.270.  $K$  was 0.40. The excretion during the next two days was too low to permit accurate calculations

TABLE 4  
*Effect of salyrgan on bromide excretion*  
Dog 2

PERIOD	DURATION	WATER INGESTED	URINE				BLOOD PLASMA			K
			Volume	Halide	Bromide	Bromide Halide	Halide	Bromide	Bromide Halide	
Before injection of salyrgan										
	hours	cc./hour	cc./hour	mM/hour	mM/hour		mM/l.	mM/l.		
4/12-4/13	24	4.2	2.9	.067	.0073	.109	102	27.5	.270	.40
4/13-4/15	48	2.5	2.2	ca. .02	ca. .004		104	28.7	.280	
5 mgm. salyrgan per kgm.										
4/15	1	0.0	48.0	13.5	2.75	.204	98	27.9	.285	.72
4/15	2	0.0	45.0	10.8	2.05	.190	96	27.5	.286	.67
4/15-4/16	21	11.9	4.2	.29	.059	.203	92	27.8	.280	.73

of the ratio. During the first hour after injection of salyrgan the total halide and bromide excretions rose to 13.5 and 2.75 mM per hour, respectively. The urinary and plasma bromide:halide ratios were 0.204 and 0.285, respectively, and  $K$  was 0.72.

Fulton and his co-workers (31) noted that salyrgan and theophylline led to an increased excretion of chloride and to a decrease in the plasma chloride. The present experiments show that this effect holds with respect to both bromide and chloride and that during the periods of diuresis induced by these substances the degree of preferential excretion of chloride, as measured by the value of  $K$ , decreases.

## DISCUSSION

It is frequently stated that the kidney does not differentiate between the chloride and the bromide ions (22, 23)—in other words, that the urinary



plasma bromide:halide ratios are practically equal (i.e.,  $K$  has a value of 1). Investigations which have been carried out on the rabbit do indeed appear to substantiate this statement (10-13); there can be found only one group of data, that of Ellinger and Kotake (14), which indicate that  $K$  has a value markedly less than 1. In contrast to the usual findings in the rabbit, investigations in the dog indicate almost unanimously that in this species the kidney excretes chloride preferentially (14-21). The data of various workers permit calculations of  $K$ , ranging from about 0.3 to 0.8.

In the present work we have found that the value of  $K$  depends upon the circumstances under which bromide excretion proceeds. It may range from 0.75 to 1 during the first hour following injections of sodium chloride or bromide or during the active portions of the diuresis induced by salyrgan and theophylline. During such periods, then, the kidney apparently is differentiating only to a slight extent between bromide and chloride. However, from 3 to 24 or 48 hours after the injection of halide, the kidney again differentiates markedly as evidenced by a value of  $K$  equal to about 0.4 to 0.5. Palmer and Clarke (21) observed a similar phenomenon under somewhat different experimental conditions. They found that in a dog receiving a normal amount of chloride in its diet, the injection of sodium chloride or bromide gave a value for  $K$  of about 0.6 to 0.7 for the first 24 hours following injection but that thereafter, for a period of as long as 4 months,  $K$  was 0.35 to 0.4.

We have also noted that the value of  $K$  depends upon other circumstances, notably the degree of halide saturation of the animal and the amount of halide injected into halide deficient animals. The possibility exists that there are other conditions, not investigated as yet, which influence the extent of the differential excretion of bromide and chloride. The question arises as to how the degree of differential excretion is related to the bromide retention. The solution to this problem may be approached in the following manner. If we multiply Equation 1:

$$K = \frac{Br_u/H_u}{Br_p/H_p} \dots \dots \dots (1)$$

by the factor  $K' = \frac{Br_p/H_p}{Br_i/H_i}$ , we obtain the following equation:

$$K = \frac{1}{K'} \cdot \frac{Br_u}{Br_i} \cdot \frac{H_i}{H_u} \dots \dots \dots (2)$$

As indicated earlier in the paper,  $Br_p$ ,  $Br_u$ , and  $Br_i$  are the amounts of bromide in the plasma per liter, in the urine, and in the food, respectively, and  $H_p$ ,  $H_u$ , and  $H_i$  are the corresponding amounts of halides.

A hypothetical instance may be used to illustrate how the degree of differential excretion of chloride and bromide is related to the extent of bromide storage on a given bromide:halide ratio in the food. Let us assume, for example, that a dog is receiving 5 mM sodium bromide and 10 mM sodium chloride daily in its food, that its extracellular body water is 3 liters, that the



serum halide concentration is 115 mM per liter of serum water and that after a certain time the urinary excretion is equivalent to the ingestion of bromide and total halide. If  $K$  which expresses the degree of differential excretion, is 0.6, then  $K'$  (or  $\frac{Br_p/H_p}{Br_i/H_i}$ ) is  $\frac{1}{0.6}$  or 1.67. Since  $Br_i/H_i$  (the food bromide: halide ratio) is 0.333, then  $Br_p/H_p = 0.55$ , or  $Br_p = 64$  mM per liter of serum water. The bromide stored in the body is then  $\frac{64 \times 3}{0.95}$  (0.95 is the Donnan factor (32)), or 202 mM. On the other hand, if  $K$  were 0.7, then the bromide stored in the body would be only 174 mM. If there were no differential excretion the bromide stored under the above circumstances would be still less,—about 100 mM. The preceding calculations are more involved if it is assumed that the urinary excretion does not become equivalent to the ingestion of bromide and halide, that is, if it is assumed that fecal excretion of bromide becomes appreciable during prolonged administration. The calculations illustrate, however, the same principle, namely that the extent of the differential excretion of bromide and chloride influences the storage of bromide in the body.

Brodie, Brand and Leshin (32) have advocated using the determination of the concentration of bromide in serum water to measure the volume of extracellular fluid in the body. This use is based on the assumptions that bromide—like chloride—is distributed extracellularly and that the amount of bromide retained in the body may be readily calculated as the difference between the amount ingested or injected and that excreted in the urine. Though there are data in the literature which indicate that the fecal excretion of bromide is negligible (14, 25), the possibility exists that under certain conditions, such as prolonged administration of bromide, the urine does not constitute the sole pathway of bromide excretion (33). In view, therefore, of the growing tendency to use serum bromide determinations in the determination of extracellular body fluid it seems advisable to investigate more fully the excretion of bromide ion from the body.

#### SUMMARY

1. The urinary excretion of bromide and chloride ions was studied in fasting dogs following (a) the intravenous injection of sodium chloride or sodium bromide (b) the administration of various diuretics.

2. The excretion of bromide or chloride ions in more than negligible amounts depended upon the concentration of total plasma halide; in general, excretion did not occur at levels below 108 mM per liter of plasma, in the absence of salyrgan or theophylline.

3. The extent of differential excretion of bromide and chloride was represented by  $K$ , the ratio of the mols per cent of bromide in the urinary halide to that of bromide in the plasma halide.  $K$  had a value ranging from about 0.7 to 1.0 during the periods of diuresis following large injections of sodium



chloride, sodium bromide and the subcutaneous injections of salyrgan or theophylline. In subsequent periods,  $K$  fell to a value of about 0.4.

4. The amount of bromide excreted following intravenous injection was related to (a) the state of halide saturation of the animal (b) the bromide:halide ratio in the plasma and (c) the value of  $K$ .

5. The relation between the bromide:halide ratios in the food intake, the plasma, and the urine has been described, and the significance of the urinary excretion of bromide for the problem of bromide storage and the calculation of extracellular water has been pointed out.

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# A METHOD FOR STUDYING EXPECTORANT ACTION IN ANIMALS BY DIRECT MEASUREMENT OF THE OUTPUT OF RESPIRATORY TRACT FLUIDS\*

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Received for publication June 2, 1941

The present investigation was undertaken with the view of obtaining quantitative information on the production and composition of liquid secretions produced in the respiratory tract, secretions which may be referred to as respiratory tract fluids (R.T.F.). Changes in the production and composition of these fluids are widely believed to be involved in the action of expectorant drugs, which, according to present therapeutic usage and theory, are drugs which are valuable in the treatment of cough because they influence the production, composition or excretion of respiratory tract fluids. Many drugs are, or have been used as expectorants with practically no information as to whether they do what they are supposed to do and even less as to how they act, if they act at all. It must be admitted that the present usage of expectorants is largely a relic of folk medicine and that while there may be many useful drugs in the compendium of expectorants there is need for a separation of the wheat from the chaff. The lack of precise information is primarily due to the lack of suitable methods of investigation. As Gunn (13) has so aptly noted, "If it were as easy to determine the quantity of bronchial secretion as it is to measure the amount of urine, there would be by this time no such vagueness as still exists."

In the present report we wish to describe a method which we have developed and adapted to laboratory animals for measuring the output and composition of respiratory tract fluids together with some figures on the output in cats and rabbits, factors which affect that output and some preliminary data on a few commonly used expectorants. Briefly, we collected fluid from a cannula placed in the trachea of a urethanized animal held head downward. A small but appreciable amount of fluid could be collected under these circumstances if the inhaled air was warmed to body temperature and saturated with water vapour. If the inhaled air was not so conditioned, no or only minute amounts of fluid could be obtained.

\*This paper was presented in part before the Canadian Medical Association at its Annual Meeting, Winnipeg, June, 1941. The work was aided by grants from the Associate Committee on Medical Research of the National Research Council, Ottawa, Canada and from the Ciba Company Limited of Montreal, Quebec.



There have been a number of previous methods employed to estimate the output of respiratory tract fluids. Probably the oldest method has been to note the effect of expectorants on the output of sputum. In recent years, Stanley Alstead of Glasgow has put this technique on a quantitative basis using patients with chronic bronchitis (1-3). He found that neither ipecac, potassium iodide, ammonium chloride or ammonium carbonate significantly affected the volume of sputum so obtained, findings which cast doubts upon common statements in textbooks of pharmacology and which also do not agree with results obtained by our method in which we have found that ipecac, ammonium chloride and ammonium carbonate increase the output of R.T.F. in animals. To explain the discrepancy, we have two explanations to offer. The first involves dosage. Using a dose of ammonium chloride corresponding on a body weight basis to that of Alstead, we also could detect no increased output of R.T.F. but using larger doses we obtained appreciable increases (see figs. 3 and 4). In the second place, our data were obtained upon approximately normal animals while those of Alstead were upon patients with chronic bronchitis. During the course of a previously reported study in which we employed patients similar to those of Alstead, we found (7) that glycerol guaiacolate (Resyl-Ciba) sometimes actually decreased the output of sputum; in the present work we found that glycerol guaiacolate increased the output of R.T.F. in animals (see fig. 4); in patients with the common cold, we have found (unpublished data) that this same drug considerably decreased the number of coughing spells. It would seem that these apparently different effects may be due to differences in the experimental animal upon which the same drug is allowed to act. Florey, Carleton and Wells (9) note that in human chronic bronchitis the bronchial mucosa consists of two general types, either desquamated epithelium or a mucosa made up almost exclusively of goblet cells which are actively secreting mucus. The data of Florey *et al.* were obtained in Great Britain presumably upon patients with histological changes in the bronchial mucosa similar in nature to those studied by Alstead. Goblet cells in the bronchial mucosa are probably not innervated and are probably capable of producing a large amount of mucus (9). Thus the large volume of mucus produced by the numerous goblet cells in the goblet cell proliferative type of chronic bronchitis might be expected to buffer and obscure any variation in the output of R.T.F. by the innervated acinar sub-mucosal glands. The effect of any expectorant which depends for its action on a reflex stimulation of the bronchial glands might therefore be obscure in the goblet cell proliferative type of chronic bronchitis. In the desquamative type of chronic bronchitis, which is probably more common in this country, the effect of a reflex expectorant could be expected to vary according to the degree of impairment of the acinar glands. In both types of chronic bronchitis there is destruction of the ciliated columnar epithelium which normally plays a major rôle in the excretion of R.T.F., and the chief excretory mechanism left is coughing which would thus have to be controlled or taken into consideration in the evaluation of any results in patients of this type.

A second method of studying the output of R.T.F. was proposed by Rossbach (16). He opened the trachea of cats and dogs, blotted areas of mucosa dry and observed the time required for the dried area to become moist again. He obtained positive results with a number of expectorant drugs but conflicting data were obtained with the same method by Calvert (6). Florey, Carleton and Wells (9) discredited results obtained by this method when they showed that vascular transudation assists in the moistening of dried areas of mucosa, a mechanism which was not affected by atropine and which would probably also obscure the effect of any expectorant which depended for its action upon reflex stimulation of the bronchial glands.

A third method was offered by Henderson and Taylor (14). They tracheotomized cats anesthetized with chloroform and urethane, arranged the animal head downward



and attached a tube containing calcium chloride to the tracheal cannula. The output of R.T.F. was measured as the increase in weight of the tube containing the calcium chloride. In criticising their method, Henderson and Taylor pointed out that they were also measuring in part the water content of the expired air, a variable that, "imposed very great difficulties which were not in every case overcome" (14). A similar procedure was used later by Schilf (17) who weighed little tubes of calcium chloride placed in the trachea and left there for varying intervals during the action of expectorants.

A fourth method of measuring the production of R.T.F. consists of measuring the water content of the respiratory tract before and at intervals after the administration of expectorant drugs, it being assumed that an increased production of a watery R.T.F. would be reflected in an increased water content of the respiratory tract. This technique



FIG. 1. PHOTOGRAPH OF THE ARRANGEMENT USED FOR COLLECTING RESPIRATORY TRACT FLUIDS

was first employed by Vollmer (18) who compared the dry weight of the lungs with that of the liver in guinea pigs. The method was further elaborated by Boyd and Johnston (4, 5) who divided the respiratory tract of albino rats into alveolar, bronchial and tracheal portions and were able to show that the water content of especially the tracheal portion could be increased by expectorant drugs. This method is free of the use of anaesthesia and of surgical manipulation, the animals being normal until they are killed and the respiratory tract dissected out. The disadvantages are that the method does not yield samples of R.T.F. which can be measured directly and analysed and that it is a slow method which requires very large numbers of animals in order to prove significant the very small differences in water content which are encountered.

A fifth method has been extensively used by Gordonoff (11). He introduced lipiodol into the respiratory tree and visualized its dilution and movements roentgenologically.



This method has the advantage that it can be applied to man but it does not yield directly measurable amounts of R.T.F. and the interpretation of some of the results seen in the films may be obscure.

A sixth method has been proposed by Florey and Wells (10) who isolated a section of the trachea *in situ*, filled it with olive oil and connected a manometer at one end to measure the dilution and increase in volume of the olive oil.

*Authors' method.* Rabbits and cats were used, anaesthetized with ethyl carbamate intraperitoneally. A side arm cannula was fastened into the trachea and the animal arranged on a board head downward on its back at an angle of about 60° with the table top. The side arm of the tracheal cannula was connected with a piece of glass tubing 20 cm. long and 4 cm. wide containing a wire tray which held wads of absorbent cotton kept continuously wet with water during the experiment. This piece of glass tubing lay within a box heated with two 40-watt bulbs. The air inhaled by the animal under these conditions was estimated to be at a temperature of 35 to 38°C. and at a relative humidity of over 80 per cent. This estimation was based upon the finding that laboratory air drawn through this conditioning apparatus at a rate similar to that at which the animals inhaled air was found, when it entered a chamber containing a thermometer and an Hampton hygrometer, to be at this temperature and relative humidity.

Respiratory tract fluids were collected in a graduated 15 cc. centrifuge tube attached to the distal and lower end of the tracheal cannula. To prevent condensation of water vapour on the cool and exposed parts of the tracheal cannula, collecting tube and glass connections, these were all insulated with thick rolls of cotton. A photograph of the arrangement is shown in Figure 1.

The experimental animal under these conditions differs from a normal animal in four respects, namely (a) it is anaesthetized, (b) it is held partially upside down, (c) it contains a foreign body in the trachea, (d) it inhales air which is artificially treated as to temperature and humidity and is probably partially rebreathed. The results which are described below do not apply directly to normal animals, therefore. The absolute output of R.T.F. so measured may differ somewhat from that in the normal animal but the relative effects of the various factors and of expectorant drugs should correspond to those in normal animals.

#### THE RATE OF PRODUCTION OF R.T.F. IN RABBITS AND CATS

The volume of R.T.F. collected in the receiving tube was noted at intervals of one hour each, beginning at the time the animal was connected to the air conditioning apparatus. The volume noted at the end of these hourly intervals was then expressed as cc. of R.T.F. per kilo body weight per 24 hours. Thus, for example, if a cat weighing 2.4 kilos put out 0.2 cc. of R.T.F. in a given period of one hour, the rate would be calculated as  $0.2 \times 24/2.4$  or 2.0 cc. per kilo per 24 hours. The hourly rate of production of R.T.F. so calculated from data obtained over a period of seven hours in 14 healthy rabbits and 8 healthy cats is shown in table 1. These do not represent selected experiments and it is obvious that there is considerable variation in the rate from animal to animal and even in the same animal over several hours. Some of the reasons for these variations will be described below and it is hoped that others will be studied later but for the time being it was found that, providing a sufficient number of animals were used, the mean rate of production of R.T.F. per hour was relatively constant over a period of



6 to 8 hours, which was the period of study in most of these experiments. The mean rate of production of R.T.F. was about 2 cc. per kilo per 24 hours in both rabbits and cats, corresponding to a total daily output of about 5 cc. for an adult rabbit or cat.

TABLE 1

*Hour-to-hour rates of production of respiratory tract fluids in urethanized rabbits and cats in terms of cc. per kilo per 24 hours*

ANIMAL NUMBER	DURATION OF EXPERIMENT—HOURS						
	1	2	3	4	5	6	7
<i>14 rabbits</i>							
11	2 26	2.26	2 26	2.26	1.70	2.80	
12	1 13	1.13	3.40	1.10	2.80	2 80	
15	0.23	1.80	1.35	1.10	1.20	1.30	1 30
16	1.00	0 80	1.10	3.60	1.20	1.70	0 23
25	0 96	1.68	1.39	6.00	2.90		
29	4.80	6.00	12.00	3.60	12 00	1.80	
32	2.40	3.60	2.40	0.00			
34	1.20	1.20	3.60	2.02	2.40	2 40	
60	2.02	2 70	2.70	3.20	2 70	2 70	1.35
62	1.06	3.20	4.20	2.00	2 13	2 13	3.20
83	1.50	0 00	2.50	2 30	3 50	2.00	1.50
113	2 30	3.00	2.30	0.00	2.30	3.00	3.00
116	1.80	0.00	0.60	0.06	1.20		
120	1.20	2.40	3.00	3.60	1 20	1 80	1.20
Mean . .	1.68	2.10	3.00	2.20	2.40	2 30	1.70
<i>8 cats</i>							
2	1.30	2.4	3.0	2.7	1 3	2.7	1.3
3	1.3	1.8	2.0	3.0	2.7	2.7	
6	1.5	1.5	1.5	2.2	2.2	3.0	3.0
11	0.0	0.7	0.0	1 0	0.7	1.35	0.7
13	3.9	2.9	2.4	4.5	2.9	1 5	1.5
16	1.0	0.3	1.4	1.0	1.0	2.0	
19	1.6	0.8	2.4	1 3	4.4	2 4	
20	2.5	1.2	1.2	3.3	3 3	0.8	2.5
Mean . . .	1.6	1.4	1.7	2.2	2.3	1 9	1.8

The question next considered was whether each animal could be used in subsequent experiments as its own control by observations made during an initial period before administering drugs, etc., and if so how long this control period should be. It will be obvious from a perusal of the data in table 1 that while a majority of the animals put out less R.T.F. in the first than in subsequent hours, variations in the mean value after the first hour were due to relatively large differences in a few animals. This applied to all hours



except the 7th when in rabbits especially the rate seemed to be falling off but as a number of animals had not survived to the 7th hour, we have neglected this 7th hour in subsequent calculations. Hourly major variations in the mean output of R.T.F. need not thus be expected after the first hour, and to illustrate this we have plotted the mean hourly output of R.T.F. smoothed by the method (8) of moving averages in figure 2. It is apparent from this figure that the output of R.T.F. during the second hour of experiment could be taken as an approximation of the control value and that if any drug administered at the end of the second hour affected the subsequent hourly output of R.T.F. by about 50 per cent or more it could probably be shown to

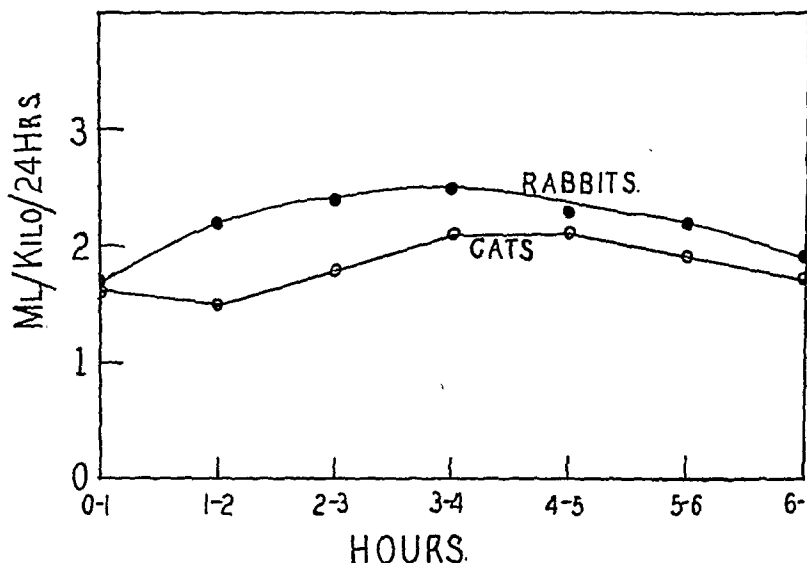


FIG. 2. MEAN RATE OF PRODUCTION OF RESPIRATORY TRACT FLUIDS IN URETHANIZED RABBITS AND CATS

have a significant effect although, of course, each case would have to be judged upon its own merits. These considerations, plus the fact that there are a number of factors which affect the absolute output of R.T.F. from animal to animal, lead us to the conclusion that it would be more satisfactory to make each animal its own control, taking as a control value the output of R.T.F. in the second or third hours, then administering an expectorant and noting any effect.

#### FACTORS WHICH AFFECT THE RATE OF PRODUCTION OF R.T.F.

1. *Temperature and humidity of inspired air.* The output of R.T.F. was found to be markedly affected by the temperature and humidity of inspired



air. These factors have not as yet been investigated in detail nor broken down but when air at 21 to 22°C. and 10 to 20 per cent relative humidity was inspired, the rate of output of R.T.F. fell to about 5 per cent of that in animals inspiring air at 35 to 38°C. and 80 to 90 per cent relative humidity. In all subsequent experiments air of the latter variety was used.

2. *Body weight.* Smaller rabbits and cats produced R.T.F. at a rate faster than that of larger rabbits and cats. Thus, for example, during the second hour of experiments on 29 rabbits and 8 cats weighing 1.5 to 2.1 kilos, the mean  $\pm$  standard error rate of production was  $2.0 \pm 0.12$  cc./kilo/24 hours while the corresponding figures in 26 rabbits and 8 cats weighing 2.2 kilos and over were  $1.5 \pm 0.12$ . This mean difference of 0.5 cc. was 2.9 times its standard deviation of 0.17 which, according to Bradford Hill (15), indicates that the odds were only about 1 in 350 that the difference was due to chance alone. The heavier animals may have produced less R.T.F. because they had relatively less tissue in the respiratory tract per kilo body weight or because they may have been older than the lighter animals; Boyd and Johnston (4) found that the respiratory tract of old albino rats had significantly less water than that of young animals.

3. *Seasonal variation.* Boyd and Johnston (4) found the trachea and lungs of albino rats to be drier in the winter months than during the rest of the year. We have similarly found that the output of R.T.F. varies with season. The mean rate of production of R.T.F. during the second hour of experiment, expressed as cc./kilo/24 hours was:

2.2 in 24 rabbits in September–October, 1940.

1.8 in 39 rabbits in November–December, 1940.

1.5 in 41 rabbits in January–February, 1941.

2.3 in 24 rabbits in March–May, 1941.

The mean difference in rate of production of R.T.F. between the rabbits examined in September–October and those examined in January–February was 0.7 and it had a standard deviation of 0.26, indicating the odds to be about 99 out of 100 that the difference was not due to chance alone. It may be concluded that the absolute output of R.T.F. decreases in the winter months.

4. *Sex.* The rate of production of R.T.F. did not vary with the sex of the animals. The mean rate was 1.8 cc./kilo/24 hours for 62 male and exactly the same for 40 female rabbits.

5. *Rate of respiration.* Gordonoff (12) concluded that respiratory movements played a major role in the excretion of R.T.F. We noted the hourly rate of respirations in some 75 of our rabbits and then plotted the output of R.T.F. against the respiratory rate. No correlation was seen to exist so that the rate of respiration alone does not appear to be a factor.



## PRELIMINARY DATA ON THE COMPOSITION OF R.T.F.

R.T.F. obtained from rabbits and cats consisted of an opaque, yellowish liquid which contained a mucus-like material and some leucocytes, red blood cells (perhaps from the surgical manipulations) and epithelial cells. The material was centrifuged and the precipitate gave a positive Benedict's test for sugar after, but not before, acid hydrolysis and was insoluble in excess acetic acid, indicating that it contained a mucin. The clear mother liquor had a mean viscosity of 1.36 as measured by the Ostwald viscosity pipette in 12 rabbits and 1.2 in 8 cats. The density of the R.T.F. was 1.0 to 1.1 in both rabbits and cats. It gave qualitative tests for chlorides, sodium, potassium, calcium, protein and when ashed yielded a test indicating the presence of minute amounts of iodine.

## THE ACTION OF EXPECTORANT DRUGS

A number of experiments were performed to see if several drugs usually regarded as expectorants affected the rate of output of R.T.F. as measured by this technique. The general procedure was to arrange the animals as described and measure the hourly output of R.T.F. for two hours, then administer the drug and see what, if any, effect occurred during the next four hours as compared with the output in the hour just before administering the drug. A probable dose of the drug to be given was tried out and if it had no effect the dose was increased until an effect was recorded or until the lethal dose was reached.

1. *Ammonium chloride*. A dose of 0.4 gm. of ammonium chloride per kilo given by stomach tube appreciably increased the output of R.T.F. in 12 rabbits. The maximal increase was registered two hours after giving the drug at which time the mean rate was 88 per cent greater than that of the control period (the hour just before giving the drug), the mean difference being 3.2 times its standard deviation giving odds of about 1 in 400 that it was due purely to chance. The secretion contained more mucus, its viscosity was greater (average increase 13 per cent) and a rough estimate of the qualitative tests suggested that it contained less chloride, sodium and potassium.

Some experiments were done to investigate the mode of action of ammonium chloride. A dose of 0.1 gm. of ammonium chloride per kilo was given intravenously to 12 rabbits; the drug produced an immediate hyperpnea and a slow increase in the output of R.T.F. reaching a maximum 5 hours after administration at which time there was a mean maximal increase in the output of R.T.F. of 106 per cent. The fact that ammonium chloride by stomach tube had proven more rapidly effective than when given intravenously suggested that the drug might be acting reflexly from the stomach, a suggestion also advanced among others by Henderson and Taylor (14). Ammonium chloride was therefore given by stomach tube to 13 rabbits in which all observable branches of the vagus nerve supply to the stomach were severed at



the gastric end of the oesophagus; in 10 of these animals there was no immediate increase in the output of R.T.F. such as had been observed with the gastric nerves intact; in the remaining 3 animals an immediate increase in the output of R.T.F. occurred but on autopsy at the end of the experiment and careful dissection of the oesophageal coat, intact nerve fibers passing to the stomach were found to have been missed in the operative procedure. The

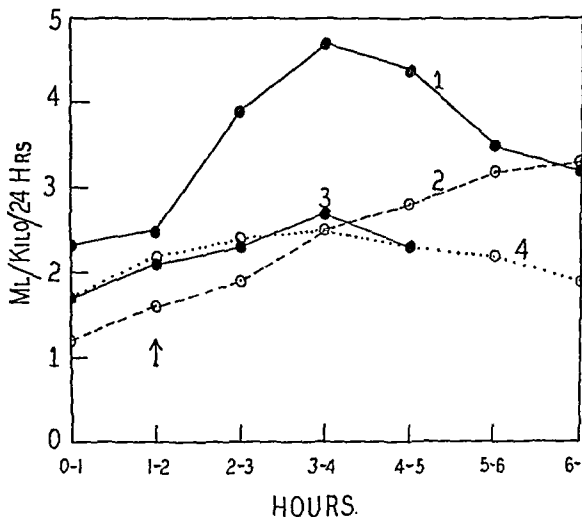


FIG. 3. EFFECT OF AMMONIUM CHLORIDE ON THE RATE OF PRODUCTION OF RESPIRATORY TRACT FLUIDS

1, administered by stomach tube, 2, administered intravenously, 3, administered by stomach tube, the gastric nerves having been cut; 4, the normal rate of production of R.T.F.

mean changes in the output of R.T.F. in these three experiments on ammonium chloride have been charted in figure 3. It is obvious that the immediate increase in the output of R.T.F. following the giving of ammonium chloride by stomach tube is dependent upon intact gastric nerves and since, as will be shown later, vagal stimulation increases the output of R.T.F. as measured by this method, the reasonable conclusion is that ammonium chloride exerts a reflex expectorant action from the stomach.



2. *Ammonium carbonate*. Ammonium carbonate given by stomach tube in a dose of 0.5 gm. per kilo to 7 rabbits also increased the output of R.T.F.,

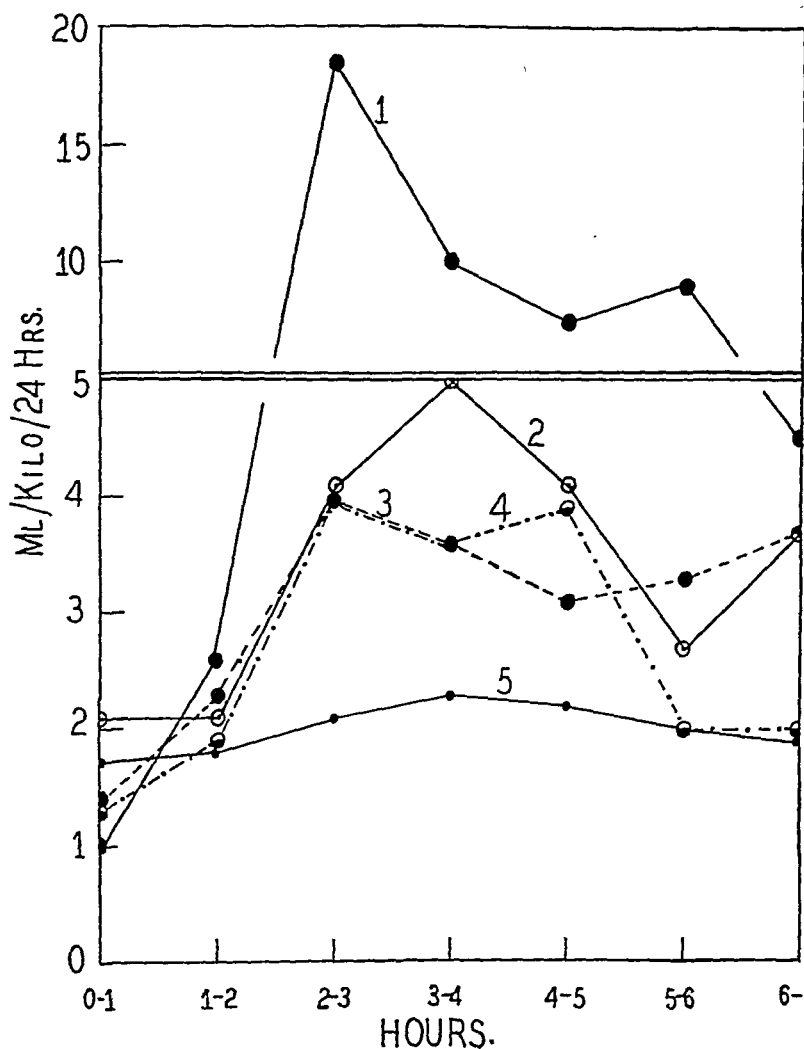


FIG. 4. EFFECT OF PILOCARPINE, IPECAC, "RESYL" AND AMMONIUM CARBONATE ON THE RATE OF PRODUCTION OF RESPIRATORY TRACT FLUIDS

1, pilocarpine; 2, ipecac; 3, resyl; 4, ammonium carbonate; 5, the normal rate of production of R.T.F.

the maximal effect occurring in one hour at which time the mean increase was 110 per cent over that of the control period (Figure 4).



3. *Powdered ipecac, B.P.* Powdered ipecac given by stomach tube suspended in saline in a dose of 1 gm. per kilo body weight to 6 rabbits increased the mean output by R.T.F. by a maximum of 43 per cent but the same dose given similarly to 9 cats increased the mean output a maximum of 143 per cent and this latter result is shown as curve 2 in figure 4. There was also more mucus in the R.T.F. secreted by the cat.

4. *Glycerol guaiacolate ("Resyl").* Connell, Johnston and Boyd (7) have reported that glycerol guaiacolate injected intraperitoneally into albino rats increased the water content of the upper respiratory tract and interpreted their results as indicating an increased output of R.T.F. We injected this

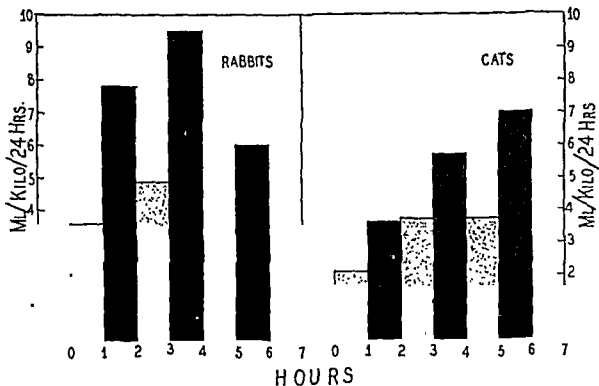


FIG. 5. EFFECT OF VAGAL STIMULATION ON THE RATE OF PRODUCTION OF RESPIRATORY TRACT FLUIDS

Results during hourly periods when the vagus was stimulated are indicated by black columns; and when not stimulated by dotted columns.

drug intraperitoneally in a dose of 0.01 gm. per kilo body weight into 11 rabbits and confirmed this suggestion of Connell, Johnston and Boyd. Mean changes in the rate of output of R.T.F. are shown as curve 3 in figure 4. The R.T.F. secreted following administration of glycerol guaiacolate was less viscous and contained less mucus.

5. *Thymol.* Thymol was given in a dose of 1 gm. per kilo by stomach tube to 4 rabbits. In two animals there was a slight increase and in two others no change in the output of R.T.F.

6. *Pilocarpine nitrate.* Pilocarpine nitrate was given intraperitoneally to 5 rabbits in a dose of 8.5 mgm. per kilo and increased the output of R.T.F.



a maximum of 73 per cent. Cats were found to be more sensitive to this drug, for a dose of 1.5 mgm. per kilo intraperitoneally raised the output of R.T.F. within the first hour by a mean of over 600 per cent in 3 cats (fig. 4, curve 1). The R.T.F. secreted by cats after pilocarpine contained much more mucus.

#### THE EFFECT OF VAGAL STIMULATION

The vagus nerve was exposed on one side of the neck and stimulated with silver electrodes and a minimal faradic current from an induction coil. The animal was arranged with the electrodes attached in place and R.T.F. allowed to collect over an initial control period of 1 hour with no vagal stimulation. Then the vagus was stimulated with currents of 5 seconds duration applied each minute over a period of one hour. In the subsequent third and fifth hours no stimulation was applied and in the subsequent 4th and 6th hours the vagal stimulation was repeated. The average results obtained in 5 rabbits and 3 cats have been illustrated in figure 5 and they demonstrate that when the vagus nerve is stimulated the output of R.T.F. is increased. The R.T.F. secreted by the rabbit after vagal stimulation contained less visible mucus, while the R.T.F. of the cat contained more mucus.

#### SUMMARY

A method is described for measuring the output of respiratory tract fluid (R.T.F.) in animals with data on cats and rabbits. The animals are anaesthetized with ethyl carbamate, a side arm cannula inserted into the trachea, the inspired air warmed to body temperature and saturated with water vapour. The R.T.F. is allowed to drain into a measuring tube and the exposed parts are insulated to prevent condensation of water vapour.

Under these conditions, rabbits and cats yield about 2 ml of R.T.F. per kilo body weight per 24 hours. The R.T.F. consists of a yellowish, opaque fluid containing mucus, chlorides, calcium, sodium, potassium, iodine compounds, leucocytes and epithelial cells.

Decreasing the temperature and humidity of the inspired air decreased the output of R.T.F. Smaller adult animals produced relatively more R.T.F. than larger adult animals and the output was decreased in the winter months. Sex did not influence the results nor did the respiratory rate.

Ammonium chloride, ammonium carbonate, powdered ipecac, glycerol guaiacolate (Resyl) and pilocarpine increase the rate of output of R.T.F. Ammonium chloride did not have this effect if given by mouth to animals with the gastric nerves severed.

Stimulation of the vagus nerve increased the output of R.T.F. in both rabbits and cats.

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# THE EFFECT OF 2,3,5, TRIODOBENZOATE AND VARIOUS OTHER COMPOUNDS ON THE GROWTH OF THE TUBERCLE BACILLUS<sup>1</sup>

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Received for publication June 5, 1941

It has been shown (1) that various substances markedly increase the oxygen uptake of a washed suspension of tubercle bacilli. These include aldehydes, alcohols, the sodium salts of fatty acids, and benzoates and salicylates. It was of interest therefore to determine the effect of these substances on the growth of the bacteria. Many other bacteria are able to oxidize aldehydes, alcohols and acids, but as far as has been determined the salicylate and benzoate effects are peculiar to the tubercle bacillus. Therefore a number of substituted benzoates and salicylates were tested on both growth and oxygen uptake of this organism. Of these compounds, 2,3,5-triiodobenzoate was the most effective in inhibiting the oxygen uptake of the tubercle bacillus in the presence of the added substrates (2) and in inhibiting growth. Its effects were therefore studied in greatest detail and an addendum at the end of the paper gives a short account of its action on various animals and man.

## EXPERIMENTAL

Both the B<sub>1</sub> and H37 strains were used. They were grown on veal-glycerine infusion broth in bottles containing 100 cc. and all the experiments were carried out on this medium at pH 6.8. In subculturing, loops of as nearly as possible the same size were taken. As the outer edges of a growing mass of tubercle bacilli comprise the youngest and consequently the most vigorous cells, care was taken in every experiment to get representative samples of all ages in the control and experimental bottles. The bottles were incubated at 37°C. and measurements of the area were taken daily. Each point in the growth curves given below represents the average of five bottles. At various times the experiments were terminated, the bottles autoclaved, and the dry weight of the bacteria determined. In general there was good agreement between area and weight. The few exceptions were caused by an unusually thin, widespread growth which was noted at the time. The pH of the drugs was adjusted to 6.8 before their addition to the medium and whenever possible the drugs were autoclaved with it. Volatile substances such as alcohols and aldehydes were added after they were passed through filters. All experiments to which significance is attached were repeated several times.

Figure 1 shows the effect of different concentrations of recrystallized triiodobenzoate on the growth of the H37 and B<sub>1</sub> strains. Marked inhibitions

<sup>1</sup> This work was aided by a grant from the Duke University Research Council.



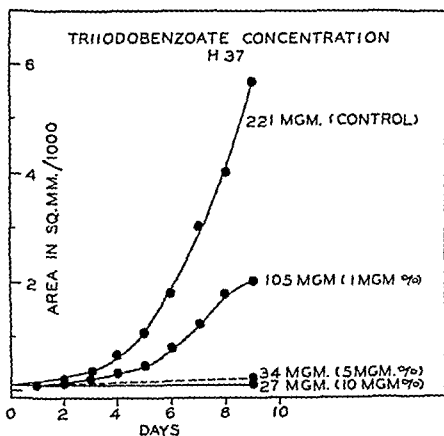
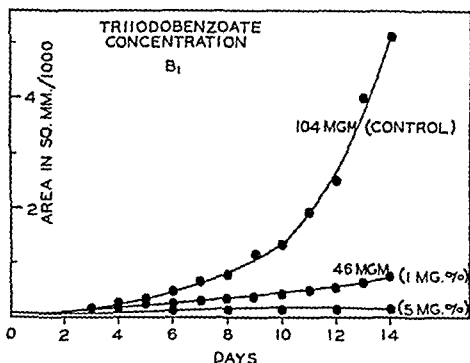


FIG. 1. THE EFFECT OF DIFFERENT CONCENTRATIONS OF 2,3,5 TRIODOBENZOATE ON THE GROWTH OF B<sub>1</sub> AND H<sub>37</sub> STRAINS OF TUBERCLE BACILLI AT PH 6.8 AND 37°

The concentration of the drug is given in mgm. per cent. The weight in milligrams is the dry weight. Each point represents the average of five bottles plotted from area measurements.



were obtained in concentration of 1.0 mgm. per cent. The addition of 10 cc. of whole blood to 90 cc. of the media did not affect the extent of the inhibition. In order to determine whether the drug was bacteriostatic or bactericidal, a culture which had not grown for three weeks in 5.0 mgm. per cent was transferred to fresh media. Growth occurred but at a rate slower than the controls. This experiment shows that the drug is merely bacteriostatic and the slower growth rate was probably due to the carrying over of

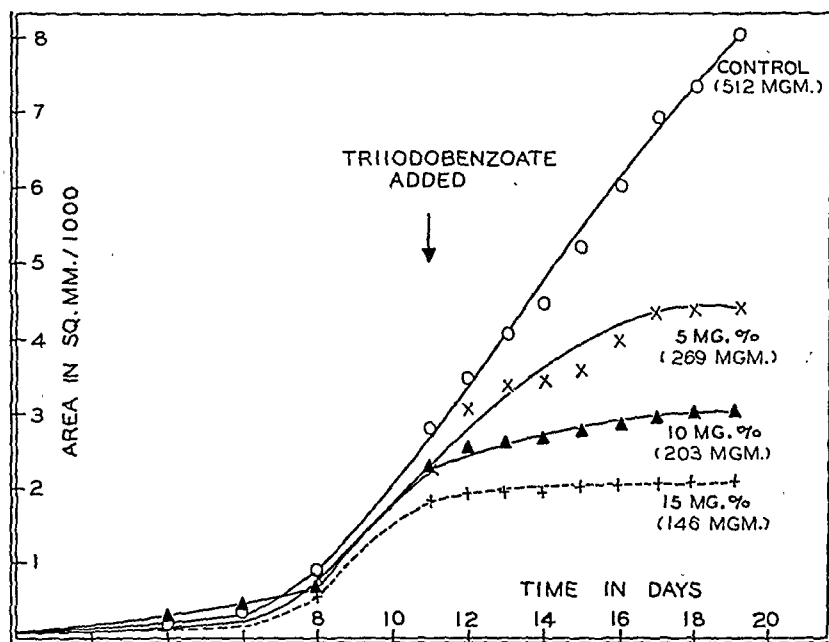


FIG. 2. THE EFFECT OF THE ADDITION OF DIFFERENT CONCENTRATIONS OF 2,3,5 TRI- IODOBENZOATE ADDED AT THE ARROW ON THE GROWTH OF THE H37 STRAIN OF TUBERCLE BACILLI AT PH 6.8 AND 37°

The concentration of the drug is given in mgm. per cent. The weight in milligrams is the dry weight. Each point represents the average of five bottles plotted from area measurements.

some of the drug. That inhibition also occurs when the drug is added during the rapid growth phase is seen in figure 2. 5.0 mgm. per cent added at this time almost completely inhibits further growth.

Table 1 summarizes the effect of a number of compounds on growth. The majority show definite inhibitions, some have no effect, and no significant accelerations were obtained despite the fact that aldehydes, salicylate, benzoate and alcohols markedly increase the oxygen uptake of tubercle bacilli. There is thus no correlation between the effects of various compounds



on growth and metabolism with the exception of triiodobenzoate and 3,5 diiodo-2-hydroxybenzoate. This latter compound, like the former, inhibits the extra oxygen uptake caused by salicylate, benzoate, etc. and is almost equally effective in inhibiting growth (table 1). It differs from triiodobenzoate in that it is bactericidal, for no growth was obtained on transferring a culture that had been exposed to a concentration of 5.0 mgm. per cent for three weeks to fresh media. Its isomer, 3,5 diiodo-4-hydroxybenzoate has little effect on the oxygen uptake of the bacilli in equivalent concentrations and requires higher concentrations to inhibit growth.

#### DISCUSSION

It is possible to make certain generalizations about the compounds that inhibit the growth of the tubercle bacillus *in vitro*. Substituted benzoic acids seem to be more effective than substituted nicotinic or sulfonic acids. The ortho isomer of sulfanilamide as indicated in the table has little effect, sulfanilamide itself requires a concentration of 50 mgm. per cent to inhibit growth, and sulfanilamide derivatives all require high concentrations (3, 4, 5). Monoiodo benzoic acids are ineffective compared to the triiodo or diiodo-2-hydroxy benzoic acids. The relative ineffectiveness of diiodo-4-hydroxybenzoic acid indicates that the presence of two iodine atoms in the ring is not in itself sufficient and that substitution in the ortho position is important. This latter conclusion is supported by the fact that salicylic acid inhibits growth whereas benzoic acid in equivalent concentrations is without effect. The substitution of salicylic acid with groups other than iodine as in dinitrosalicylic acid, *o*-methoxybenzoic acid, thiosalicylic acid etc. usually decreases the inhibitory action of the compound. The substitution of three groups into benzoic acid in itself is without effect as shown by the inactivity of trimethoxybenzoic and triaminobenzoic acids the latter probably being converted to phloroglucinol.

#### SUMMARY

1. The effect of substituted benzoic and nicotinic acids on the growth of the H37 and B<sub>1</sub> strains of tubercle bacilli has been studied.
2. The most effective compounds were 2,3,5 triiodobenzoate which was bacteriostatic and 3,5 diiodo-2-hydroxybenzoate which was bacteriostatic and bactericidal.

#### ADDENDUM

*L. Mims and F. R. Johnston*

2,3,5 triiodobenzoate injected intraperitoneally in doses of 500 mgm. per kilogram into rats and guinea pigs caused no toxic symptoms. 100 mgm. per kilogram injected daily for two weeks intraperitoneally into rats caused



TABLE 1

The dry weight in milligrams of *B<sub>1</sub>* and H37 strains of *tubercle bacilli* grown for the indicated times in the presence and absence of various compounds on *yeast-glycerine infusion broth* at pH 6.8 and 37°

Each figure represents the average of five cultures. NG signifies no growth. Some differences in the rate of growth of the controls were noted with different batches of media and the age of the inoculum. This variation did not significantly effect the percentage inhibitions. Some apparent accelerations listed below were not significant because of the scattering of the results.

STRAIN	COMPOUND	DATE OF GROWTH	CON- TROL	1.0 MG. PER CENT	5.0 MG. PER CENT	10.0 MG. PER CENT	15.0 MG. PER CENT	STRAIN	COMPOUND	DATE OF GROWTH	CON- TROL	1.0 MG. PER CENT	5.0 MG. PER CENT	10.0 MG. PER CENT	15.0 MG. PER CENT
H37 B <sub>1</sub>	2,3,5 triiodobenzoate	9 14	221 104	105 46	34 NG	27 NG		H37	nicotinic acid	13	169	224			239
H37 B <sub>1</sub>	3,5 diiodo-2-hydroxy benzoate	17 11	199 83	157 33	43 33	NG 36	NG NG	H37	nipeptic acid	13	169	206			183
H37 B <sub>1</sub>	3,5 diiodo-4-hydroxy benzoate	12 12	224 140	218 104	149 65	132 67	93 NG	H37	6-hydroxynicotinic acid	13	169	198			282
H37 B <sub>1</sub>	o-iodobenzoate	15 18	441 158	510 137	510 137		115 97	H37	α-amino nicotinic acid	10	247	140			213
H37 B <sub>1</sub>	p-iodobenzoate	7 18	210 158	199 131			178	H37	quinolinic acid	10	247	278			344
H37 B <sub>1</sub>	m-iodobenzoate	14 18	363 158	262 137	262 137		181 97	H37	2-hydroxynicotinic acid	16	260	291			310
H37 B <sub>1</sub>	o-bromobenzoate	8 18	266 158	297 149	283 149	294	270 145	H37	picolinic acid	10	247	188			52
H37	p-bromobenzoate	10	362	399	200	72	72	H37	o-aminobenzeno sulfon- ic acid	9	175	119			108
H37	m-bromobenzoate	10	362	491	425	467	344	H37	2,4,6 triaminobenzoate	12	707	526			657



H37	<i>p</i> -chlorobenzoate	8	277	262	168	163	151	H37	3,4,5 trimethoxybenzoate	12	707	557	700
H37 B <sub>1</sub>	<i>o</i> -chlorobenzoate	15 18	441 158		419 154		400 158	H37	isoamylalcohol	14	116	153	149
H37 B <sub>1</sub>	salicylate	10 15	334 42	213 28	72 NG	53 NG	76 NG	H37	isovaleraldehyde	14	116	218	168
H37 B <sub>1</sub>	thio-Salicylate	9 13	175 140		166 134		133 105	H37	benzaldehyde	10	316	369	402
H37 B <sub>1</sub>	dinitro-Salicylate	9 13	175 140		76 118		86 130	H37	anisaldehyde	10	316	285	270
H37	<i>o</i> -methoxybenzoate	16	260		253		290	H37	furfural	13	175		82
H37	2,4 dihydroxybenzoate	16	260		317		298	B <sub>1</sub>	<i>o</i> -aminobenzene sulfonamide	15	178	169	152
H37	benzoate	14	116		151		170						
H37	<i>o</i> -nitrobenzoate	16	172		268		197						
H37	<i>m</i> -dimtrobenzene	16	172		64		NG						
H37	$\beta$ -methoxyphthalate	16	172		167		111						



no weight loss and microscopic examination of the tissues showed no damage in the liver, kidney and heart. The same dose intraperitoneally into guinea pigs caused weight loss and some liver damage but given by mouth showed no toxic action. *In vitro* experiments (6) showed that triiodobenzoate did not inhibit the metabolism of liver and kidney and only inhibited that of brain in concentrations of 50 mgm. per cent.

When 1.0 gram of the drug is taken by mouth by a 70 kgm. man it is excreted in the urine slowly. The excretion was followed with Kendall's (7) iodine method. The iodine was all present in organic combination indicating that the molecule, which is stable to boiling with acid and alkali, is not broken down in the body to liberate iodide. The excretion after a 1.0 gram dose rises slowly to a maximum between the third and fifth day and some is still being excreted on the tenth day. In all about 60 per cent was recovered in the urine. The rest may be stored in the body or excreted by the intestine. 0.5 gram three times a day for ten days has been given to an 11 kgm. child without apparent toxic effects, as judged by histological examination of the kidney, liver and spleen.

3,5-Diiodo-2-hydroxybenzoate has not been studied in detail. It seems somewhat more toxic to guinea pigs.

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# STUDIES ON THE TOXICITY AND PHARMACOLOGY OF PANTOTHENIC ACID

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Received for publication June 11, 1941

The group of chemically identified B-vitamins which comprises thiamine, riboflavin, nicotinic acid and pyridoxine, has recently been augmented by a new member, pantothenic acid. This substance has been identified as a derivative of  $\beta$ -alanine (1), namely, *N*-( $\alpha$ - $\gamma$ -dihydroxy- $\beta$ , $\beta$ -dimethyl-butyryl)- $\beta$ -alanine (2); it has since been synthesized (3) and made available in the form of its dextrorotatory calcium salt.

Pantothenic acid was first recognized as a growth factor for yeast by R. J. Williams (4), who coined the name pantothenic acid (meaning ubiquitous) because it was found to be present in a great variety of foodstuffs. The vitamin nature of this substance was discovered simultaneously by Jukes (5) and Woolley *et al.* (6), who found that "chick dermatitis," an experimental deficiency disease, can be cured by the addition of pantothenic acid to the diet. The rôle of pantothenic acid in nutrition of rats has been studied by several investigators (7-12). In young rats, lack of pantothenic acid causes failure to grow, impairment of the fur, inflammation of the nose and hemorrhages, particularly into the adrenal cortex. Black rats maintained on diets free from pantothenic acid develop a conspicuous greying of the hair; the depigmentation is prevented or cured by the administration of pantothenic acid (13, 14). Rats which receive pantothenic acid in addition to the other crystalline factors of the B-complex reach maturity and are reproductive (15, 16).

In this study crystalline dextrorotatory calcium pantothenate has been used. This substance is readily water soluble and almost neutral in reaction, the pH of a 10 per cent solution being approximately 8. All experiments reported here were performed on animals maintained on the usual laboratory diets.

## ACUTE TOXICITY

Acute toxicity was studied in mice, rats, dogs and monkeys.<sup>1</sup> A dose of 1 gram of calcium pantothenate per kgm. body weight was fed to each of 5

<sup>1</sup> A preliminary report on the toxicity of pantothenic acid has appeared in the *Proc. Soc. Exp. Biol. & Med.*, 45: 311, 1940.



dogs and to one monkey. No toxic symptoms were observed in these animals. After 2 weeks the monkey and one dog were sacrificed; examination of the organs failed to show any pathologic changes.

In mice and rats the L.D.50 was determined following oral, subcutaneous, intraperitoneal and intravenous administration, 10 animals being used for each dose level. Table 1 summarizes the results obtained from a total of 190 rats and 230 mice.

Rats dosed with 10 grams per kilogram of calcium pantothenate by mouth survived without showing toxic symptoms. Lethal doses produced prostration and respiratory failure in rats and mice. Death occurred within 2 hours

TABLE 1  
*Acute toxicity of calcium pantothenate*  
L.D. 50. (Gram per kilogram body weight)

	ORAL	SUBCUTANE- OUS	INTRAPERI- TONEAL	INTRAVENOUS
Mice..... (20-22 grams)	10.0	2.7	0.92	0.91
Rats..... (150-200 grams)	>10.0	3.4	0.82	0.83

TABLE 2  
*Comparison of the acute toxicity of nicotinic acid, pyridoxine and pantothenic acid in rats following oral and subcutaneous administration*  
L.D. 50. (Gram per kilogram body weight)

	ORAL	SUBCUTANEOUS
Sodium nicotinate.....	7.0	5.0
Pyridoxine hydrochloride.....	5.5	3.7
Calcium pantothenate.....	>10.0	3.4

following intravenous and intraperitoneal injection, and within 6 to 12 hours following oral and subcutaneous administration. No late deaths were observed.

The low toxicity of pantothenic acid agrees well with the low toxicity of other members of the vitamin B-complex. As shown in table 2, its acute toxicity is of the same order of magnitude as that found for nicotinic acid (17) and pyridoxine (18).

#### CHRONIC TOXICITY

Chronic toxicity was studied in rats, dogs and monkeys. Daily doses of 50 and 200 mgm. respectively of calcium pantothenate were fed to young



male and female rats over a period of 190 days. The animals showed normal development and their growth did not differ significantly from that of a control group (table 3). Autopsies at the end of the feeding period did not reveal any gross or microscopic changes in the organs. Offspring of the group receiving 50 mgm. of calcium pantothenate daily were given the same daily dose of pantothenic acid as soon as they were weaned. These animals (2nd generation) likewise developed normally and their weights increased at the same rate as those of the control group (table 3).

Six adult dogs were fed 50 mgm. per kilogram of calcium pantothenate daily over a period of 6 months, and 4 monkeys of 4 to 5 kgm. body weight received 1 gram of calcium pantothenate daily over the same period of time. Both dogs and monkeys failed to show any toxic symptoms, their weight

TABLE 3

*Growth of young rats receiving calcium pantothenate daily by mouth*

	AVERAGE WEIGHT—GRAMS							
	At start		20 days		50 days		120 days	
	Males	Fe- males	Males	Fe- males	Males	Fe- males	Males	Fe- males
Control. Stock diet only (10 males, 10 females)	46	44	104	98	208	168	314	228
200 mgm. calcium pantothenate daily (10 males, 10 females)	45	44	96	87	202	154	308	210
50 mgm. calcium pantothenate daily (10 males, 10 females)	44	44	98	95	203	158	312	210
50 mgm. calcium pantothenate daily, 2nd generation (4 males, 6 females)	46	44	120	97	210	152	304	212

either remained constant or increased. All animals were sacrificed at the end of the feeding period. Histopathological examination failed to reveal any changes.

#### LOCAL EFFECTS

Local effects of calcium pantothenate were studied on rabbits by subcutaneous injection and instillation into the conjunctival sac. No irritation, inflammation or abscess formation were observed in 8 rabbits following the subcutaneous injection of 1.0 cc. of 1, 2, 5 or 10 per cent solutions of the vitamin. The infiltration of the subcutaneous tissues subsided about as rapidly as that following an injection of 1.0 cc. of saline. Instillation of 0.5 cc. of a 10 per cent solution into the conjunctival sac of 3 rabbits did not produce any irritation.



*Effect on metabolism*

The action of pantothenic acid on the metabolism of rats was studied by the method of Richards and Collison (19). In 16 experiments in which calcium pantothenate was given orally in doses of 1 and 50 mgm. respectively, no change in oxygen consumption was found on observing the animals for three hours after the administration of the vitamin, although it is rapidly absorbed. In studies on the absorption and excretion of pantothenic acid (20) the peak of its concentration in the blood was observed within 2 hours following oral administration.

The effect of pantothenic acid on water metabolism was studied in 30 rats of 180 gram average body weight. The diuresis of groups of 5 rats was fol-

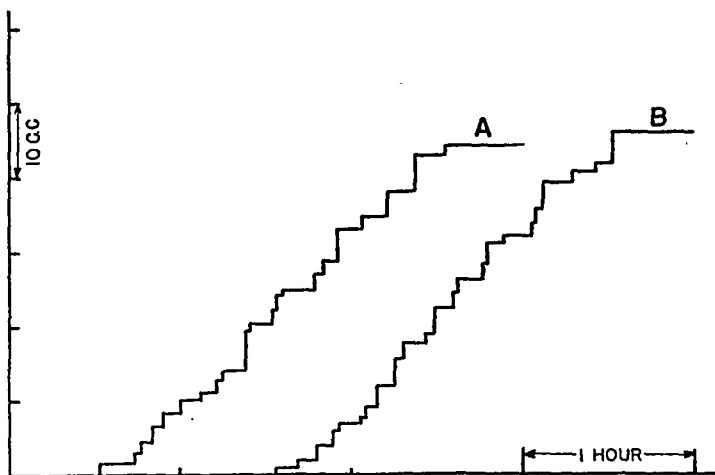


FIG. 1. CONTINUOUS RECORDING OF THE DIURESIS OF 5 RATS FOLLOWING A DOSE OF 5 CC. OF TAP WATER PER 100 GRAMS BODY WEIGHT

A. Water alone. B. Water and 50 mgm. calcium pantothenate per rat

lowed using the diuresis recorder of Kniazuk (21). The normal excretion following 5 cc. of tap water per 100 grams body weight was first established for each group. A dose of 50 mgm. of calcium pantothenate given simultaneously with the water by mouth or by intraperitoneal injection did not influence either the rate or the volume of urine excreted (fig. 1).

*Effect on circulation*

The blood pressure and the respiration of cats under urethane and chloralose anesthesia were not influenced by intravenous injections of calcium pantothenate in doses ranging from 10 to 50 mgm. per kilogram. The heart rate, as measured by a new recording device (Kniazuk and Unna, 22), remained unchanged (fig. 2).



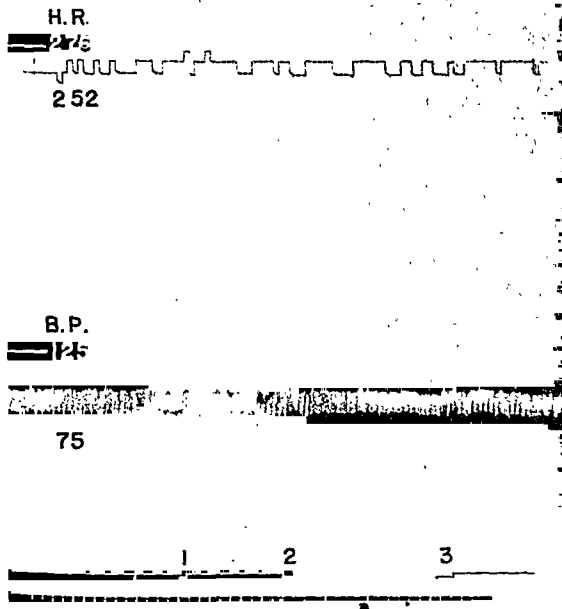


FIG. 2 EFFECT OF PANTOTHENIC ACID ON RESPIRATION, HEART RATE AND BLOOD PRESSURE

Cat 3.5 kgm. body weight. 0.8 gram per kilogram urethane and 0.06 gram per kilo-  
gram  
per



*Effect on Smooth Muscles*

On the isolated intestine and isolated uterus of the rabbit, calcium pantothenate in concentrations up to 1:10,000 had no visible effect. Spasms of the intestine induced either by barium chloride or acetyl choline were not influenced by pantothenic acid.

## ACKNOWLEDGEMENT

Appreciation is expressed to Dr. W. Antopol for the histological studies and to Mr. S. Kuna for valuable technical assistance.

## SUMMARY

1. The acute toxicity of calcium pantothenate has been determined in mice and rats following intravenous, intraperitoneal, subcutaneous, and oral administration. The L.D.<sub>50</sub> following subcutaneous injection is 2.7 grams per kilogram in mice and 3.4 grams per kilogram in rats.

2. Daily administration of calcium pantothenate over a period of 6 months to monkeys (1 gram per monkey), dogs (50 mgm. per kilogram) and rats (50 and 200 mgm. resp. per rat) failed to produce any toxic manifestations or pathological changes in the organs.

3. The metabolism, the circulatory and respiratory systems, and the smooth muscle organs of normal animals are not influenced by calcium pantothenate.

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# STUDIES ON THE RELATION OF DRUG ADDICTION TO THE AUTONOMIC NERVOUS SYSTEM: RESULTS OF COLD PRESSOR TESTS

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Received for publication June 13, 1941

Not only do certain of the signs and symptoms of the morphine abstinence syndrome indicate a disturbance of the autonomic nervous system (1), but the impression has been gained that autonomic reactions to ordinary stimuli may be sufficiently disagreeable to persons who become addicts to cause them to use a drug which alters the reaction pattern to unpleasant stimuli (2). The present studies were undertaken with the view that improved prevention and treatment of drug addiction might result from a better understanding of the relation of the autonomic nervous system to the nature and etiology of the condition.

It seemed desirable to investigate this by employing a method which would distinguish variations in reaction to a stimulus that disturbs a peripheral function controlled by the autonomic nervous system. Since studies by Light and Torrance (3) have shown the cardio-vascular system *per se* to be unaffected by drug addition, it seemed safe to employ the cold pressor test of Hines and Brown (4) for this purpose. This test, based on the vasoconstriction reflex, consists of measuring the rise in systolic blood pressure caused by a standard cold stimulus and the time required for recovery after its removal.

*Comments on the significance of the cold pressor reaction.* Hines and Brown first considered 15 mm. Hg as the maximal normal reaction to the cold pressor test (4); later the limit was raised to 22 mm Hg (5). Persons without hypertension who gave responses greater than 22 mm. Hg, termed "normal hyperreactors," included patients with Raynaud's disease, effort syndrome, and hyperthyroidism.

The following observations by Hines and Brown (3) serve to elucidate the reaction: Interruption of the blood flow from the immersed hand did not affect the response. Barbiturates and alcohol reduced the response, bismuth subnitrate had no effect, and calcium chloride increased it. The pressor effect of cold was abolished by ether anesthesia and diminished somewhat by spinal anesthesia. Cervicothoracic or lumbar ganglionectomy, or both, had no effect on the response, but bilateral extirpation of the seventh through twelfth or thirteenth thoracic ganglia (dog) reduced it. Bilateral adrenalectomy (dog) resulted in no alteration of the pressor response except when cortical extract was withheld. Patients with Addison's disease gave positive responses.

These observations would appear to indicate that the pressor response to cold (vasoconstriction) is a neurogenic reflex mediated through and perhaps modified by higher (vasomotor) centers, and effected, in part at least, through the thoracic sympathetic



ganglia. In this connection it is of interest to note that Marquis and Williams (6), in studying the vasoconstriction response to painful stimuli (including cold) in normal persons and patients with discrete lesions involving the sensory pathway, found it to vary directly with the intensity of the stimulus and with changes in subjective appreciation of the stimuli. Lesions of the sensory tracts of the thalamus or cerebrum did not appear to affect the response. These authors concluded that the ascending pathway for the response is the spino-thalamic tract; and that the reflex is complete in the brain-stem below the thalamus. However, experimental evidence for cortical areas (4 and 6 Brodmann) influencing vasomotor reactions has been recorded by Pinkston and Rioch (7). Observations recorded by Bronk, Pitts, and Larrabee (8) would appear to confirm the finding of Marquis and Williams, for the response to peripheral stimulation was not affected by removal of the hypothalamus. Under appropriate experimental conditions, however, stimulation of the hypothalamus was shown to cause the blood pressure to rise; *concomitant peripheral stimulation augmented this effect.*

#### METHODS

The *basal systolic level (B.S.L.)* was estimated by making blood pressure determinations, while patients rested in the supine position, until three consecutive readings at five minute intervals varied by not more than 2 mm. Hg. Then the contralateral hand was placed in ice water for one minute and the rise in systolic pressure was noted, the last reading being made just before or as the interval timer rang. The highest value noted during this period is called the *Ceiling*, and the difference between B.S.L. and Ceiling is termed the *Response*.

The hand was then removed from the ice water, dried, and wrapped in a towel, and blood pressure determinations were continued at one minute intervals until the basal systolic level was regained, or for four minutes after withdrawal of the hand. The term *Recovery* is used to denote the time in minutes until the systolic blood pressure fell to or within 2 mm. Hg of the B.S.L.

These tests were made on post-addicts, on normal controls, and on addicts before and after withdrawal of morphine. All subjects were white adult males without hypertension or known cardiovascular disease. The "post-addicts" (patients from whom drugs had been withdrawn at least 9 months previously) were divided into two groups: Group I was composed of 44 patients chosen at random, whereas Group II comprised an equal number of patients tested within the 24 hours (average of 8 hours) prior to discharge from the institution. The average periods of incarceration of these groups were 19.3 and 18.8 months respectively.

The addicts tested were patients admitted directly to the hospital with definite physical dependence on an opiate. Tests were made while they were stabilized on the minimal amounts of morphine required to prevent signs of withdrawal. After withdrawal of morphine, tests were made at regular intervals through the sixth month of total abstinence on all patients that remained under treatment and who were not suffering from some intercurrent illness.

The control subjects were doctors and attendants. The consistency of the reaction was checked by testing 7 normal controls at monthly intervals for 7 consecutive months.

Studies were made of the effects of the following drugs on the cold pressor reaction: Benzedrine, epinephrine, acetyl-beta-methylcholine, Prostigmin,<sup>1</sup> normal saline, aspirin, Nembutal, a new synthetic analgetic drug,<sup>2</sup> morphine, and strychnine.

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<sup>1</sup> Material furnished through courtesy of the Hoffman-La Roche Company.

<sup>2</sup> 1-methyl-4-phenyl-piperidine-4 carbonic acid ethyl ester hydrochloride, known as "Dolatin" in Europe, and referred to as "D-140" in this paper, was furnished through the courtesy of the Winthrop Chemical Company.



## RESULTS

Data on the addiction-abstinence phase of this study are presented in figure 1 and table 1 along with the results of the cold pressor tests made on normal controls and the two groups of post-addicts.

## COLD PRESSOR TESTS

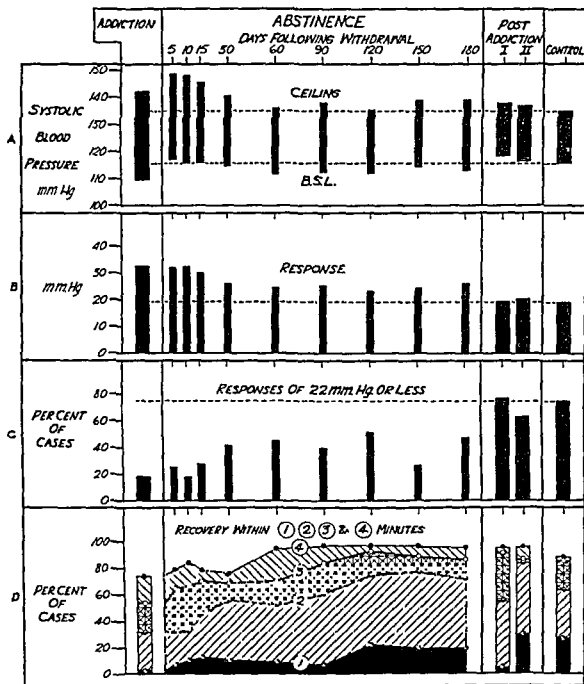


FIG. 1

Diagram A of figure 1 shows the group mean data on B.S.L. and Ceiling at each period of study with the control values extended as dashed lines. The



reaction to cold is greater than normal during addiction and early abstinence, and returns slowly toward the control range.

In diagram "B" the above data have been arranged with all B.S.L. values at 0 to show the trend in Responses from definite hyperreaction during addiction to control values in postaddiction. The percentages of patients in each group showing "normal" responses (22 mm. Hg or less) are shown in diagram "C," as evidence of the consistency of the data.

The data on Recovery are plotted in diagram D to indicate the percentage of cases in each period showing recovery within 1, 2, 3, and 4 minutes. The

TABLE 1  
*Cold pressor tests*

	ADDICTION	ABSTINENCE (DAYS FOLLOWING WITHDRAWAL)									POST ADDICTION		CONTROL
		5	10	15	30	60	90	120	150	180	I	II	
Number of cases.....	65	53	50	46	38	33	30	27	26	21	44	44	41
Mean age.....	39.4	40	39.7	40	39.8	39.3	38.5	38.4	37.8	37.7	37.7	40.6	32.9
Mean diastolic b.p.....	68	72	71	74	72	70	70	71	72	71	74	72	72
Mean rest (min.).....	28	28	28	28	29	30	30	31	28	28	30	28	30
Basal systolic level:													
Mean.....	109.5	116.8	115.8	115.7	114.6	111.7	112.6	112.0	114.5	113.0	118.6	116.5	115.9
$\sigma$ (dis.).....	8.5	9.6	7.4	9.1	10.0	8.6	9.7	6.6	9.1	6.5	8.6	8.4	8.9
$\sigma$ (av.).....	1.1	1.3	1.1	1.3	1.6	1.5	1.8	1.3	1.8	1.4	1.3	1.3	1.4
Ceiling:													
Mean.....	142.2	148.7	148.1	145.5	140.5	136.1	138.0	135.2	139.1	139.2	137.9	137.2	135.2
$\sigma$ (dis.).....	12.8	14.2	12.0	11.4	11.2	11.8	8.7	9.4	11.1	12.1	12.4	11.8	12.5
$\sigma$ (av.).....	1.6	1.9	1.7	1.7	1.8	2.1	1.6	1.8	2.2	2.6	1.9	1.8	2.0
Response:													
Mean.....	32.7	31.9	32.3	29.8	25.9	24.4	25.4	23.3	24.6	26.2	19.3	20.7	19.3
$\sigma$ (dis.).....	11.2	11.4	10.9	9.5	9.0	9.8	5.5	7.1	8.6	12.1	8.4	7.1	7.8
$\sigma$ (av.).....	1.4	1.5	1.5	1.4	1.5	1.7	1.0	1.4	1.7	2.6	1.3	1.1	1.2
Recovery:													
Per cent in 1 minute...	3.0	6.9	10.0	13.0	10.5	9.1	6.6	22.2	19.3	19.0	4.5	29.5	26.8
Per cent in 2 minutes...	29.2	25.1	22.0	32.6	44.7	42.4	53.4	51.9	57.7	52.4	50.0	54.5	36.6
Per cent in 3 minutes...	21.6	32.8	34.0	24.0	13.2	18.2	23.4	18.5	11.5	14.3	38.5	2.3	22.0
Per cent in 4 minutes...	20.0	13.8	18.0	8.7	7.9	24.2	13.3	3.7	7.7	9.5	4.5	9.1	2.4

data points during abstinence were connected to facilitate visualization of the trend of progressive change from slowed recovery during addiction and early abstinence to normal recovery by the fourth month after withdrawal. The group of post-addicts tested on the day of discharge showed more rapid recovery than the group tested at random.

*Effect of certain drugs on the cold pressor test.* The results of studies of the acute effects of certain drugs on the cold pressor test are presented in table 2. The effect of benzedrine was to raise the B.S.L. more than the Ceiling, hence to reduce the Response of both normal controls and of addicts stabilized on



morphine. Recovery was accelerated by benzedrine. Epinephrine, in the dose employed, caused no significant change in the reaction, but tended to delay Recovery. Physiological saline had no effect.

Acetyl-beta-methylcholine reduced the B.S.L. and Ceiling to the same extent, leaving the Response unchanged. This result agrees with the finding of Engle and Binger (9) on the effects of "Mecholyl" in hypertensive patients.

TABLE 2  
*Effect of certain drugs on the cold pressor reaction*

Number	SUBJECTS Kind	MEDICATION			INTERVAL	B.S.L.		CEILING		RE- SPONSE		EFFECT ON RECOVERY (+, 0, or -)*
		Drug	Dose	Route		Before	After	Before	After	Before	After	
			mgm.									
8	Controls	Benzedrine	10	Per os	2 hours	117	125	141	144	24	18	+
7	Controls	Benzedrine	20	Subq.	1 hour	112	139	131	153	19	14	+
3	Addicts	Benzedrine	10	Subq.	1 hour	105	119	131	138	29	19	0
6	Controls	Epinephrine	0.2	Subq.	30 sec.	118	118	134	136	18	18	-
7	Controls	0.85% NaCl	1 ml.	Subq.	30 sec.	118	118	134	134	18	18	0
7	Controls	Mecholyl	5	Subq.	5-19 min.	117	113	137	133	20	20	0
7	Controls	Prostigmin	1	Subq.	40 min	118	117	140	138	22	21	0
5	Controls	Aspirin	600	Per os	30 min	110	110	131	128	21	18	0
9	Post-addicts	D-140	100	Subq.	1 hour	119	115	138	126	19	11	+
8	Post-addicts	Nembutal	100	Subq.	1 hour	118	113	137	130	19	17	0
10	Post-addicts	Strychnine	2	Subq.	1 hour	113	112	131	127	18	15	0
9	Controls	Morphine SO <sub>4</sub>	10	Subq.	1 hour	115	115	133	128	18	13	+
					6 hours		114		129		15	+
8	Post-addicts	Morphine SO <sub>4</sub>	10	Subq.	1 hour	111	113	143	138	32	23	+
					6 hours		107		133		28	+
15	Post-addicts	Morphine SO <sub>4</sub>	20	Subq.	1 hour	111	115	134	129	23	14	+
					6 hours		111		125		14	0
					24 hours		111		131		20	0
					48 hours		111		131		20	+
4	Addicts	Morphine SO <sub>4</sub>	1 stab. dose†	Subq.	1 hour	107	103	133	128	26	25	+
4	Addicts	Morphine SO <sub>4</sub>	2 stab. dose†	Subq.	1 hour	112	106	137	127	27	23	+

\* + = faster, 0 = no change, - = slower recovery.

† 1 stabilizing dose = minimal amount needed to satisfy physical dependence.

Prostigmin did not significantly alter the reaction. Neither cholinergic drug affected Recovery.

Acetyl-salicylic acid did not affect the B.S.L. or Recovery, but the Response was slightly reduced by it. "D-140," a new synthetic analgetic, reduced B.S.L., Ceiling, and Response and tended to accelerate Recovery. Nembutal acted to reduce the B.S.L., Ceiling, and Response.

Neither B.S.L. nor Recovery were significantly affected by strychnine, but Ceiling and Response were reduced slightly by this drug.



The effect of morphine in addicts, post-addicts, and normal controls was to reduce the Ceiling and Response and to accelerate Recovery. This effect was slight in addicts receiving amounts of morphine just adequate to the maintenance of physiological equilibrium, and was not increased much by twice the amount. In post-addicts and normal controls the Response was reduced 28 per cent by 10 mgm. morphine; 20 mgm. morphine caused a greater reduction. This effect was still present at the end of 6 hours but had practically disappeared after 24 hours.

Tests performed at monthly intervals for 7 months on 7 normal controls appear to confirm the finding of Hines and Brown (4) as regards the constancy of the reaction and its freedom from the effects of conditioning. These data are presented in table 3.

TABLE 3  
*Reliability of the cold pressor test*  
(Mean data on seven normal controls)

MONTH (1939)	B.S.L.	CEILING	RESPONSE
February.....	119	140	21
March.....	114	132	18
April.....	119	138	19
May.....	120	136	16
June.....	118	137	19
July.....	118	140	22
August.....	116	136	20
Mean of monthly means.....	118	137	19
Mean deviation.....	1.4	2	1.4
Greatest deviation.....	6	8	6

#### DISCUSSION

The "hyperreactor" type of Response found during addiction would appear to confirm the impression that physical dependence on opiates is associated with disturbed autonomic reaction. However, the progressive improvement from the second through the sixth months of abstinence and the normal reaction of post-addicts would seem to indicate that recovery, although slow, is certainly adequate. Accordingly, if the hypothesis that the etiology of addiction and relapse is associated with functional abnormality of the autonomic nervous system be true, another perhaps more sensitive or specific test must be employed to demonstrate it.

It is understandable that aliphatic hypnotics, anesthetics and various analgetics including morphine tend to reduce the Response, for these substances reduce either the reception of peripheral stimuli, or the reaction pattern to them, or both (2). But the association of a hyperreaction with addiction to a substance which ordinarily reduces the Response would suggest that



physical dependence might constitute a part of the body defense to the continued effects of morphine; an exaggeration of normal physiological effort to maintain homeostasis in the constant presence of an agent having the tendency to alter the precise states balanced and checked by the autonomic nervous system. The experimental situation wherein the pressor response to peripheral stimulation was augmented by simultaneous stimulation of the hypothalamus (8) suggests a possible explanation of the hyperreaction to cold during addiction, namely, that this portion of the diencephalon becomes hyperirritable as physical dependence to morphine develops.

The results of this study tend to confirm in part the theory of Tatum, Seevers, and Collins (10) that addiction represents a state of hyperirritability of certain portions of the nervous system resulting from the tendency of the stimulant effects of repeated doses of morphine to be cumulative because the depressant effects are of shorter duration. While hyperirritability of a portion of the nervous system is thought to be associated with hyperreaction to cold during addiction, no evidence of hyperreaction was encountered as an acute effect of morphine.

The second group of post-addicts was tested to study the effect of emotion on the test. While the situation just prior to discharge is known to be fraught with emotional possibilities, its only effect on the cold pressor test was to accelerate Recovery.

#### CONCLUSIONS

The blood pressure response of addicts to a standard cold stimulus is greater than normal and recovery is slower than normal. This abnormal reaction, suggesting that hyperirritability of autonomic centers is associated with addiction, slowly reverts to normal in both particulars following withdrawal of morphine.

The acute effect of morphine in normal persons and in post-addicts is to reduce the blood pressure response to cold and to accelerate recovery. In this, the effect of morphine resembles the action of neither adrenergic nor cholinergic drugs, but is similar to the effect of hypnotics, analgetics, and anesthetics.

The technical assistance of the following personnel of the Research Department of this Hospital is gratefully acknowledged: Henry Elam, Charles Keebaugh, Ward Workman, Robert Blake, and Clinton Gray.

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# PAIN THRESHOLD MEASUREMENTS IN THE DOG

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Received for publication July 3, 1941

The Hardy-Wolff method for measuring the pain threshold in man has proved so useful (1) (2) that an extension of this technique to animals would be highly desirable. The success of this method in man depends to a large extent upon the distinctive subjective experience when the stimulus exceeds the sensory threshold to pain. This experience is so definite and so sharply defined that it would seem that there should be demonstrable objective phenomena accompanying it. It has been found that in man the galvanic skin reflex resulting from a stimulus strong enough to evoke a response of "pain" is considerably greater than that obtained when the stimulus is reported "hot" (unpublished data). This led to an extension of the method to animals where subjective reports are not available.<sup>3</sup>

## METHODS

A standard Hardy-Wolff apparatus having a variable intensity light source and a fixed 3 second exposure time was used. The current for the lamp was supplied from the A. C. mains through a voltage regulating transformer and was controlled by a Variac. The intensity of the stimulus was measured either by the standard radiometer technique of Hardy and Wolff or by a wattmeter connected in the lamp circuit. The latter method would not be suitable for longtime studies where blackening of the lamp might introduce serious errors, but is quite satisfactory for studying the effects of single doses of drugs. Careful measurements showed that there is a linear relation between the readings of the wattmeter and the radiometer so that either can be used in the calculations.

Two adult female dogs (each weighing 23 kgm.) were used. The hair was closely clipped over the mid-dorsum at the thoracolumbar region and this spot was thoroughly blackened with India ink. The dogs were trained to lie quietly with the blackened area directly behind the aperture, in the position customarily occupied by the forehead of the human subject in similar experiments. Stimuli were given every 30 seconds (the same interval used with humans).

## RESULTS

Attempts to record a galvanic skin reflex in the dog were not successful but we found that there was a characteristic reflex twitch of the musculature of

<sup>1</sup> Associate physicist.

<sup>2</sup> Head guard attendant.

<sup>3</sup> Since this work was started D'Amour and Smith have reported the use of radiant energy in studying the pain sensation in rats. *THIS JOURNAL*, 72: 74-80, 1941.



the back whenever a definite level of stimulation was exceeded. This twitch is not confined to the site of stimulation but includes a rather large surrounding area. Before this level was reached the dogs showed signs of discomfort following a stimulus. They would extend a front paw or attempt to rise but could readily be persuaded to return to position. This period of discomfort apparently corresponds with the point in human experiments at which a sensation of strong heat is experienced. When the stimulus reaches threshold the twitch begins just before the end of the stimulus period. This was shown by recording muscle action potentials from a surface electrode just at the margin of the stimulated area. It was found that a burst of action potentials occurred just before the end of those stimuli which were barely strong enough to elicit the twitch. In man the sharp twinge of pain is experienced at the end of the stimulation period if the stimulus is at threshold intensity. The twinge of pain in man and the reflex twitch in the dog can be elicited before the end of the stimulus, but only if the stimulus intensity is significantly above threshold. All results reported here are threshold values.

It is important that the dog be kept from touching the diaphragm through which the stimulus is received, for a similar reflex twitch can be produced by touching or stroking the skin.

The conditions required to elicit this twitch so closely parallel those required to obtain a report of "pain" in man that there can be little doubt that the same mechanism is involved.

1. The intensity of the stimulus required to evoke a response is constant to just about the same degree as in man. In dog No. 1, 53 trials showed a mean threshold at 296 watts with extremes at 330 and 260 watts. With dog No. 2, 24 trials gave values of 279, 290, and 260 watts respectively. It is interesting that the level required is of the same order as that of the pain threshold in man. The mean threshold of 70 trials on 4 men was 266 watts.

2. The same threshold is obtained when the position of the area stimulated is changed.

3. The threshold is practically independent of the size of the area stimulated.

4. With a constant area of stimulation the threshold depends quite strongly on the time of stimulation. The intensity-time relationship is quite similar to that obtained in man.

5. The threshold changes with the administration of drugs in a manner quite analogous to the changes found in man.

Figure 1 shows the threshold raising effect of 3 opiates; morphine, dilaudid, and codeine. The temporal course of the threshold-raising effect of morphine is somewhat different from that found in man but there is no reason to suppose that the metabolism of morphine is identical in the two species. As in man, the threshold-raising effect of a single dose of morphine is reasonably duplicated by a similar dose given several days later and the magnitude of the effect increases with the size of the dose.



Dilaudid shows the same rapid and intense threshold raising action as in man but the duration of effect is shorter. Codeine appears to be a much weaker drug than morphine as is the case in man, but here again the duration of effect is shorter.

Figure 2 shows the threshold raising effect of 3 non-opiates. Aspirin shows a decided threshold-raising action which is somewhat shorter and less intense than that found in man for an equal dose, without considering weight differences. A dose of nembutal sufficient to produce definite hypnosis showed no threshold-raising effect. This is in accord with results found in man, in whom

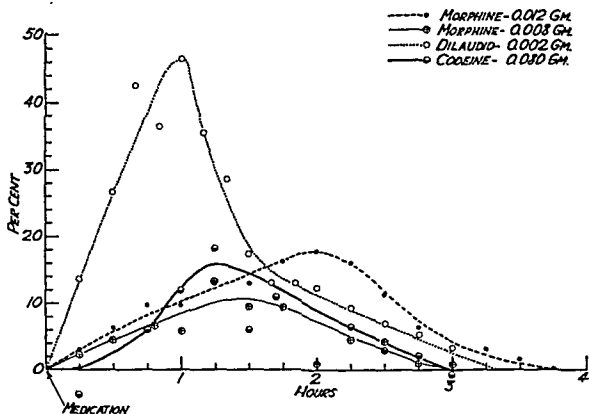


FIG. 1. THE THRESHOLD RAISING EFFECTS OF 3 OPIATES

The per cent rise in threshold is plotted as ordinates. While the temporal course of action is not the same as in man, the relative effects of the drugs are quite comparable.

the hypnotic effects of barbiturates are out of proportion to the threshold raising effects.

Prostigmin has no threshold-raising action in man and none was found in the dog, using a comparable dose. Since the primary action of prostigmin is peripheral, this lack of threshold raising action indicates that the controlling factor in the twitch response is more central, and that this is not appreciably affected by prostigmin.

To test the threshold raising effect of cobra venom (*Naja*) 1 cc. (10 mouse units) was injected intramuscularly. The threshold-raising effect of the first dose could not be studied because a severe thunderstorm soon after the injection.



tion made the dog quite unmanageable. This dose was repeated daily for 8 days and measurements were taken at 15 minute intervals for at least two

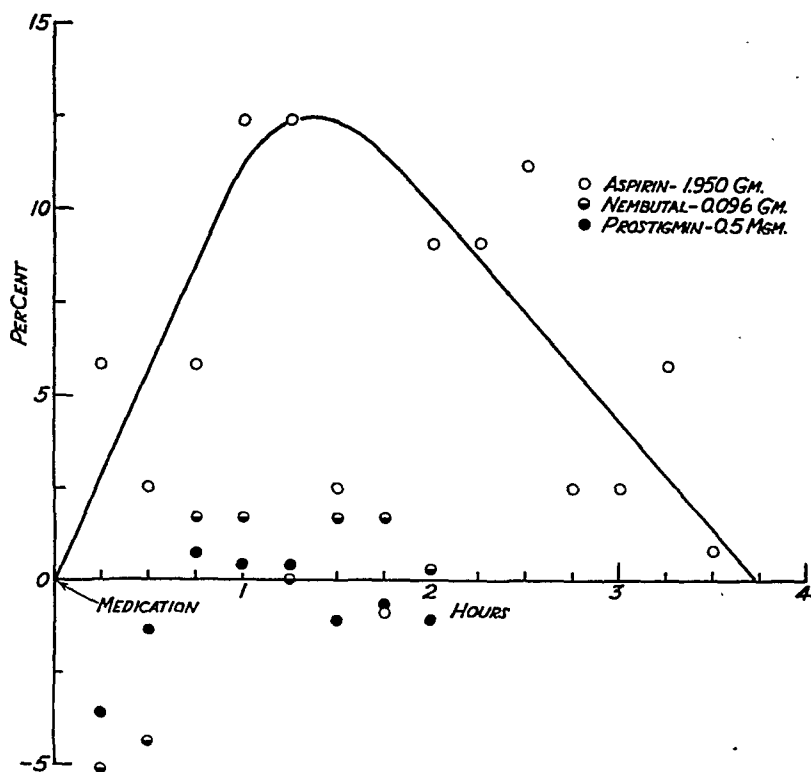


FIG. 2. THE THRESHOLD RAISING EFFECTS OF 3 NON-OPIATES  
The dose of nembutal was sufficient to produce definite hypnosis

TABLE 1

DAYS OF ADMINISTRATION	TOTAL DOSE (M. U.)	PREINJECTION THRESHOLD
1	0	295
2	10	305
3	20	310
4	30	304
8	70	311

hours after subsequent injections. There was no threshold raising effect of any of the doses studied, and the preinjection threshold showed no significant rise, table 1.



## CONCLUSIONS

The results described show that there exists in the dog a mechanism which can be used to measure pain thresholds in a manner quite analogous to that used in man. Drugs affect these thresholds in much the same way as in man and hence it seems that this method should be generally useful for studying the pain mechanism and the action of drugs on it.

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# QUANTITATIVE COMPARISONS OF THE ACTIVITY OF SULFANILAMIDE, SULFAPYRIDINE, SULFATHIAZOLE AND SULFADIAZINE AGAINST ESCHERICHIA COLI IN VIVO AND IN VITRO<sup>1</sup>

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Received for publication July 12, 1941

## I. IN VIVO

Few reports (1, 2, 3, 4, 5) have appeared on the therapeutic activity of the sulfonamide drugs in experimental infections with *E. coli* in mice. With one exception (3), the virulence of the culture used for the infection has been enhanced by using mucin (6). Although all of these reports indicate a more or less favorable effect from treatment with sulfanilamide (*p*-aminobenzene-sulfonamide), sulfapyridine (2-sulfanilamidopyridine), sulfathiazole (2-sulfanilamidothiazole) and/or sulfadiazine (2-sulfanilamidopyrimidine), the data given do not allow any quantitative assessment of the comparative activity of these drugs. Klinefelter (4) concluded that sulfathiazole was more effective than sulfapyridine in the treatment of experimental colon bacillus infections in mice and that both of these drugs were more effective than sulfanilamide. In a second communication Klinefelter (5) claimed that sulfadiazine was more effective than sulfathiazole in treating this infection in mice.

The data given in Klinefelter's first paper suggested that one dose of drug per day for three days was much more effective than administration of larger amounts of drug using the drug-diet method (7, 8). This was entirely different from what had been found in the case of streptococcus and pneumococcus infection (8, 9). In view of this discrepancy a detailed investigation to determine the most effective method of treatment of *E. coli* infections in mice seemed warranted. The results obtained, as well as a quantitative comparison of the effectiveness of certain sulfonamide drugs<sup>2</sup> are given in the present communication.

<sup>1</sup> This investigation has been aided by a grant from The John and Mary R. Markle Foundation.

<sup>2</sup> We wish to thank Eli Lilly and Co. for the sulfanilamide, the American Cyanamid Co. for the sulfapyridine and sulfadiazine, and E. R. Squibb and Sons for the sulfathiazole used in this work.



### Methods

A stock culture of the strain of *Escherichia coli*<sup>\*</sup> used in this study was subcultured daily in buffered-peptone-dextrose broth (10). Cultures for the experimental infection were obtained by transferring 0.2 cc. of a 16 hour stock culture into 9 cc. of broth. After 5 hours incubation at 37°C. broth dilutions were made in ten-fold multiples, out to 10<sup>-8</sup>. Plate counts of the 10<sup>-7</sup> and 10<sup>-6</sup> dilutions were averaged to determine the approximate number of bacteria per unit volume of diluted culture. One part of the 10<sup>-6</sup> broth dilution was mixed with nine parts of a sterile 5 per cent solution of mucin containing 2 per cent dextrose. Infection was produced by intraperitoneal inoculation of 0.5 cc. of this bacterial suspension in mucin. The infecting dose contained, on the average, 20,000  $\pm$  4000 bacteria. Five out of 115 control mice survived, the remainder died between 7.5 and 23 hours with an average survival time of 11 hours.

Experiments in which the drug-diet method was used were carried out as previously described (8); otherwise, drugs were administered orally by tubing with a blunt needle. Sulfanilamide was given suspended in a solution of acacia; the other three drugs in solution as the sodium salts. Initial doses were given in 0.5 cc. and subsequent doses in 0.25 cc. of fluid. The blood concentrations of drugs were determined as already described using 0.02 cc. of blood, diluted 1:200 for analysis (11, 8). All blood concentrations represent free and not total drug and all dosages are expressed in terms of the anhydrous drug (not sodium salts or their hydrates).

In experiments designed to determine the most effective method of drug administration, sulfathiazole was used. Mice of the CFCW strain of 15 to 20 grams weight were used throughout. All surviving mice were observed for 14 days after infection.

### Results

*Treatment with drug-diet method.* In experiments with either 0.5 or 2.0 per cent of sulfathiazole in the diet (1 to 3 days feeding prior to infection) the therapeutic effect on the colon bacillus infection was erratic. It was noted that, unlike experimental infections with streptococcus or pneumococcus injected in broth, the intraperitoneal injection of *E. coli* suspended in mucin produced immediate prostration which lasted until death of the animal. Measurement of the food intakes after infection indicated that the majority of these mice ate extremely little. An occasional mouse which ingested the drug-diet after the infection and hence received treatment survived. A few other mice also survived, presumably because a high blood level of drug was present at the time of infection. In a control experiment, mice were injected intraperitoneally with sterile mucin. These mice exhibited almost as severe prostration as the mice which had received mucin plus *E. coli*. After injection of sterile mucin the average food intake per mouse was 0.06 for the first 4 hours, 0.09 for the first 8 hours, and 0.45 gram for the first 12 hours. The infected mice consumed even less food. In contrast to this, normal mice or mice infected with streptococcus or pneumococcus ingest five to ten times this amount of food for similar periods.

*Comparison of a single dose with three daily doses.* Since a majority of deaths in treated mice occurred within 24 hours after infection with *E. coli*

<sup>\*</sup> We wish to thank Dr. H. F. Klinefelter for supplying this strain.



it seemed probable that a single dose might be as effective as daily repetitions of this dose. The data presented in table 1 indicate that this is true.

*Duration of optimum treatment.* The above data (table 1) show that the duration of treatment necessary for optimal therapeutic effect is 24 hours or less. Since a single dose of sulfathiazole (administered *per os* in solution as the sodium salt) produces a maximum blood concentration in one-half hour or less, and since repeated dosage every two hours is necessary to maintain a constant blood concentration, (see fig. 1), the following experiments were designed to determine the duration of blood concentration for optimal therapeutic response.

TABLE 1  
*Comparison of one day with three days treatment*

DAYS OF TREATMENT	ORAL DOSE OF SULFATHIAZOLE			
	1 mgm.	2 mgm.	4 mgm.	8 mgm.
1	0 (15)*	20 (15)	53 (15)	100 (20)
3	0 (14)	7 (15)	60 (15)	95 (19)

\* First figure gives per cent survival; figure in parentheses refers to number of mice used.

TABLE 2  
*Therapeutic effect of sulfathiazole with different dosage schedules*

TIME OF 1ST DOSE (4 MGm.)	2ND DOSE 2 HOURS AFTER 1ST	NUMBER OF MICE	PER CENT SURVIVAL
	mgm.		
With infection.....	0	40	33
One-half hour before infection.....	0	40	25
With infection.....	2	40	56
One-half hour before infection.....	2	40	38

In the first experiment, a comparison was made of the therapeutic effects which were obtained with four different dosage schedules: (1) a single dose at the time of infection (high blood concentration one-half hour following infection); (2) single dose one-half hour before infection (high blood concentration at time of infection); (3) first dose at time of infection followed by second dose two hours later (blood concentration as in (1) but duration longer); (4) first dose one-half hour before infection followed by second dose two hours after the first (blood concentration in (2), but duration longer). The results are given in table 2.

These data show that: (1) giving the first dose of drug one-half hour before infection is less efficient than administering the first dose at the time of infection; (2) two doses (maintained blood level) are more effective than a single dose.



A comparison was then made, using two groups of 20 mice each, of the therapeutic effect from administration of a single dose (4 mgm.) with that from smaller but repeated doses (2 mgm. initial plus three doses of 1 mgm. at two-hour intervals). The single dose (high blood concentration of short duration) produced 20 per cent survival whereas the repeated doses (lower blood concentration of longer duration) produced 60 per cent survival.

Next, it was found that the duration of the blood concentration necessary for most effective treatment was 14 to 16 hours. The data of these experiments are summarized in table 3.

TABLE 3  
*Relation of therapeutic effect to length of treatment*

DOSEAGE	NUMBER OF SUBSEQUENT DOSES AT 2 HOUR INTERVALS	NUMBER OF MICE	PER CENT SURVIVAL
Initial: 1 mgm. Subsequent: 0.5 mgm.	1	20	10
	2	20	10
	3	20	40
	4	20	75
Initial: 0.5 mgm. Subsequent: 0.25 mgm.	3	20	0
	4	20	0
	5	20	15
	6	20	40
	7	19	37

TABLE 4  
*Effect of the same amount of sulfathiazole administered in a single dose and in five doses*

	TOTAL DOSE		
	6 mgm.	3 mgm.	1.5 mgm.
Single dose.....	45*	15	25
Divided doses† .....	85	80	20

\* Per cent survival. Twenty mice used for determination of each figure.

† Initial dose:  $\frac{1}{2}$ ; subsequent doses  $\frac{1}{2}$  of total dose at 2-hour intervals.

Finally, a comparison was made of the therapeutic effect from the same amount of sulfathiazole administered in a single dose and in five doses (at two-hour intervals). In the divided dose experiment one-third of the dose was given at the time of infection and one-sixth every two hours for 4 subsequent doses. The results given in table 4 show again that a long duration of a low blood concentration is more effective than a short duration of a high blood level.

*Comparison of therapeutic activity.* The experiments described in the preceding sections establish the experimental basis for a method of comparing



the activity of drugs in a colon bacillus infection in mice. It has been shown that: (1) the drug-diet method of therapy is unsatisfactory because mucin prevents adequate ingestion of the diet for 12 out of the 14 hours during which

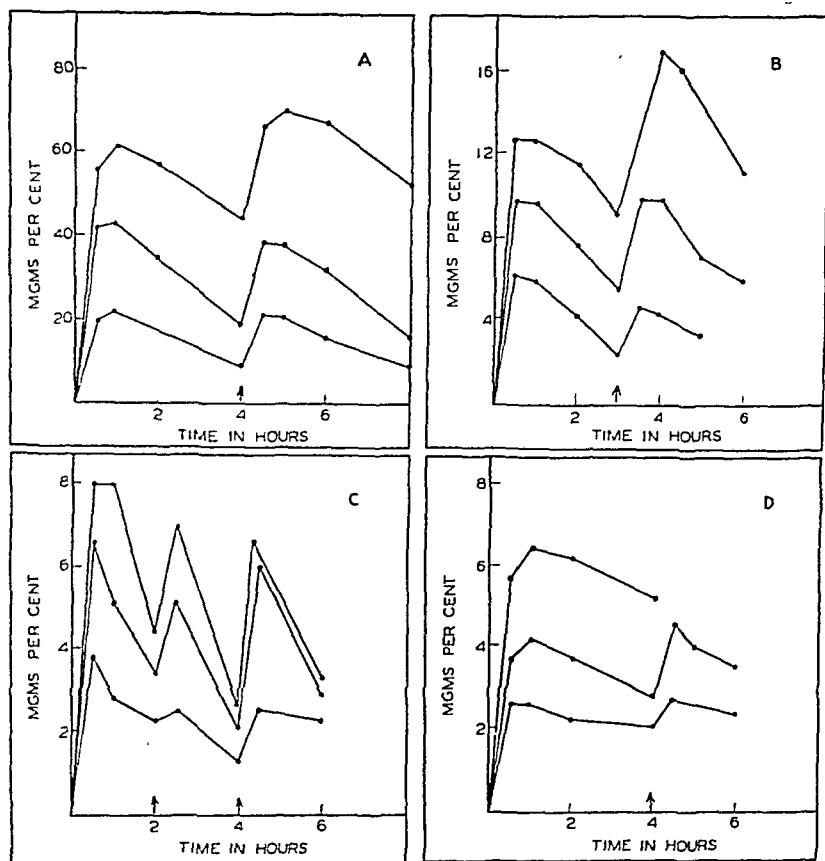


FIG. 1. BLOOD CONCENTRATION-TIME CURVES OF SULFONAMIDE DRUGS ADMINISTERED *per os* TO MICE ACCORDING TO VARIOUS DOSAGE SCHEDULES

In each case, initial dose at zero hours followed by one-half this dose at times indicated by arrows. Each curve is an average of 4 to 6 individual curves, each determined in a single mouse. A, sulfanilamide, upper, middle and lower curves, initial dose 24, 12 and 6 mgm.; B, sulfapyridine, initial dose 4, 2 and 1 mgm.; C, sulfathiazole, initial dose 2, 1, and 0.5 mgm.; D, sulfadiazine, initial dose 0.6, 0.3, and 0.15 mgm. respectively.

therapy can be achieved; (2) repeated doses of a drug are more effective than a single dose; (3) repetition of dosage so as to maintain a constant blood concentration for 12 to 14 hours is sufficient for maximum therapeutic response; (4) the therapeutic response is directly dependent upon both the



height and the duration of the blood concentration of drug; (5) more or less constant blood concentrations can be maintained by administration of drugs *per os* at appropriate times and in sufficient dosage.

Due to differences in absorption and excretion, it was necessary to determine for each drug the dosage schedule which would best maintain a constant blood concentration. In figure 1 are shown the blood concentration-time curves given by the dosage schedules used for each of the four drugs. The dosage schedules from which these curves were obtained were used in our

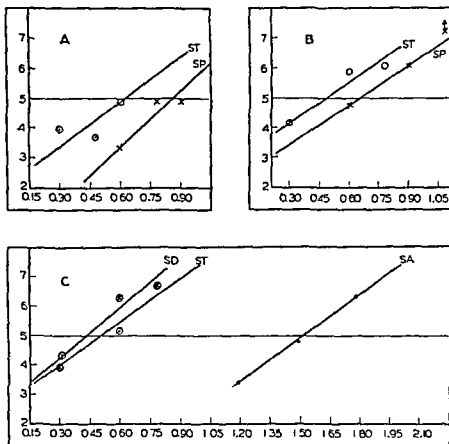


FIG. 2. RECTIFIED DOSAGE-SURVIVAL CURVES FOR SULFONAMIDE DRUGS FOR *E. coli* INFECTION IN MICE

At SA, 1 thro A, B in probits. The lines on 20 mice.

therapeutic experiments. These were: for sulfanilamide and sulfadiazine, an initial dose at the time of infection followed by one-half this dose at 4 and 8 hours; for sulfapyridine, an initial dose plus one-half this dose at 3, 6, and 9 hours; and for sulfathiazole, an initial dose plus one-half this dose at 2, 4, 6 and 8 hours. It was decided by inspection of these curves (fig. 1) that the average blood concentration maintained by the above dosage schedules were (in milligrams per cent): for sulfanilamide, 15, 30, and 60; for sulfapyridine, 4, 8 and 12; for sulfathiazole, 2, 4 and 6; and for sulfadiazine, 2, 4



and 6. In a few instances other dosage schedules were used and blood concentrations were estimated by interpolation.

Our comparison of the therapeutic activity of the four drugs is based upon four separate experiments. In the first three the per cent survival of groups

TABLE 5  
*Comparison of activity of drugs*

EXPERIMENT	DRUG	$\beta \pm \text{S.E.}^*$	S.B.C. <sub>50</sub> †	ACTIVITY RATIO	STANDARD ERROR OF LOGARITHMS OF	
					S.B.C. <sub>50</sub>	Activity ratio
A	Sulfathiazole	5.0 $\pm$ 2.0	4.3	1.0	0.045	0.056
	Sulfapyridine	6.2 $\pm$ 2.0	7.3	0.59	0.033	
B	Sulfathiazole	4.5 $\pm$ 1.2	3.1	1.0	0.049	0.076
	Sulfapyridine	3.6 $\pm$ 1.2	4.6	0.71	0.062	
C	Sulfathiazole	4.8 $\pm$ 1.2	3.2	1.0	0.046	0.065
	Sulfanilamide	4.9 $\pm$ 1.0	33.0	0.10	0.045	
	Sulfadiazine	5.7 $\pm$ 1.2	2.7	1.18	0.039	0.061

\* Signifies slope constant plus or minus its standard error.

† Signifies median survival blood concentration in milligrams per cent.

TABLE 6  
*Comparison of activity of drugs*

DRUG	BLOOD CONCENTRATIONS	PER CENT SURVIVAL†	S.B.C. <sub>50</sub>	ACTIVITY RATIO	STANDARD ERROR OF LOGARITHMS OF	
					S.B.C. <sub>50</sub>	Activity ratio
	<i>mgm. per cent</i>					
Sulfathiazole.....	4	47	4.1	1.0	0.047	0.069
Sulfapyridine.....	8	73	6.0	0.69	0.050	
Sulfanilamide.....	30	30	38.6	0.11	0.049	
Sulfadiazine.....	4	50	4.0	1.04	0.047	
Sulfathiazole*.....	2	30	2.5	1.0	0.049	0.081
Sulfanilamide*.....	15	10	27.7	0.09	0.064	

\* Blood concentration maintained for 14 to 16 hours instead of 10 to 12 hours.

† Based on 30 mice in each case.

of 20 mice each was determined for three blood concentrations of each drug maintained for 10 to 12 hours. The data of these three experiments are shown in figure 2, where blood concentration expressed logarithmically is plotted against per cent survival expressed in probits. A free hand straight



line was fitted to each set of points. The values given in table 5 were obtained by graphic solution of these curves by a method recently described (12).

A fourth experiment was carried out in which the four drugs were compared simultaneously (10 to 12-hour therapy). In addition, sulfanilamide and sulfathiazole were compared on the basis of 14- to 16-hour therapy (optimal length of treatment). In this experiment, the per cent survival of a group of 30 mice for each drug was determined at a single blood concentration which was expected to give about 50 per cent survival. The S.B.C.'s were then obtained by use of the following equation:

$$\log S.B.C._{50} = \log x + \frac{5.0 - y}{B}$$

where  $x$  is the blood concentration tested,  $y$  is the probit value of the per cent survival obtained, and  $B$  is the weighted mean slope constant of the first three experiments<sup>4</sup> (table 5). The results of this comparison are given in table 6.

The weighted mean activity ratios with their standard errors of the four experiments (tables 5 and 6) taking sulfathiazole as a standard (1.0) are: sulfadiazine  $1.08 \pm 0.11$ , sulfapyridine  $0.61 \pm 0.049$ , and sulfanilamide  $0.10 \pm 0.009$ .

## II. IN VITRO

Several reports (4, 5, 13, 14, 15, 16, 17, 18, 19, 20) have been published on the *in vitro* activity of sulfanilamide, sulfapyridine, sulfathiazole and sulfadiazine against *E. coli* in various media. In all cases, however, determinations and comparisons of *in vitro* activity have been based on methods which measure the degree of inhibition produced by the drugs, i.e., comparisons have been based on the relative degree of "response" produced by the same amount of each drug. Obviously, such methods can provide data from which only qualitative comparisons may be made. In order to obtain *in vitro* data on which a quantitative comparison could be based, we have used a method which measures the activity of drugs in terms of the smallest concentration (the "end-point") of each drug which was required to produce a "fixed response."

End-points (on which activity ratios were based) against the colon bacillus were easily obtained in a synthetic medium in which the antibacterial action of each drug was apparently favored to the utmost extent.

In order to determine whether these ratios had broad application to *in*

<sup>4</sup>The variations in the slope constants of the first three experiments (table 5) are within experimental error. The weighted mean slope constant was obtained by using the reciprocals of the squared standard errors of the slope constants as weighting coefficients. This value,  $B$ , was found to be  $4.88 \pm 0.49$ .



*vitro* activity, it was necessary to test the drugs in various kinds of media. It seemed probable that with our method of titrating drug activity, the addition to the synthetic medium of any specific anti-drug factor or any growth stimulant would change the end-point (activity) of each drug. It has been shown that the *in vitro* activity of sulfonamide drugs can be blocked by *p*-aminobenzoic acid (21), methionine (22) and a factor contained in certain extracts (23, 24). Drug activity has also been shown to be affected by certain products of protein hydrolysis (25). Therefore, we have measured and compared the activity of the four drugs in the present study under various conditions obtained by adding anti-drug factors and protein hydrolysates to a synthetic medium.

### Methods

The inhibitory activity of the four drugs was measured against three strains of *E. coli* in various media. One strain (Ward) was recently isolated from a stool culture; another (MacLeod) was obtained through the courtesy of Dr. C. M. MacLeod of the Rockefeller Institute; the third strain (Klinefelter) was that used in our experimental infection in mice. Stock cultures of these strains were transferred daily in the synthetic medium (containing glycerol) described by MacLeod (24) and in our PD broth (10). Over a period of several months, it was found that the use of PD stock cultures gave the same results as those obtained with the synthetic-glycerol media.

Test inoculums were obtained by suitable dilution of stock culture in synthetic medium. The inoculum used routinely was selected to give an initial concentration of about 1000 bacteria per cubic centimeter of test mixture.

Concentrated drug solutions were made by weighing each drug into each test medium and heating at 100°C. to dissolve. Five cubic centimeters of each concentrated drug solution was then pipetted through a series of two-fold dilutions in 5.0 cc. amounts of the appropriate medium. The initial solution of each drug was made up in a concentration which, after dilution and inoculation, permitted titration of activity over a range of concentrations increasing successively, in two-fold steps, from 0.01 mgm. per cent up to a concentration limited by drug solubility. Sulfanilamide, for example, was titrated in a series of concentrations from 0.01, 0.02, 0.04, 0.08, 0.16, and so on, up to 655.36 mgm. per cent. Our titration scale was thus made up of a power series in which the successive concentrations of drug could be represented (in milligrams per cent  $\times 100$ ) as exponents of 2. On this basis, the series of concentrations of sulfanilamide (in milligrams per cent  $\times 100$ ) could be expressed as follows:  $2^0$ ,  $2^1$ ,  $2^2$ ,  $2^3$ ,  $2^4$ , and so on, up to  $2^{16}$ . After autoclaving, each tube in the various sets of drug concentrations (together with control tubes of medium without drug) was inoculated with 0.2 cc. of diluted culture.

Turbid growth in the control tubes and in the tubes at the lower end of the range of drug concentrations, coupled with absence of visible growth at the upper end of the range, provided a sharp end-point for each titration. These inhibitory end-points were usually read after 48 hours incubation at 37°C. except in tests with media containing Tryptose peptone in which case rapid growth made it necessary to read end-points at the 24th hour. Although the end-points changed in any one test medium with continued incubation, the relative activity of the drugs, i.e., the ratios, remained constant (within experimental error) for at least 96 hours. Under the conditions of our tests heavy growth occurred in from 8 to 18 hours in the control tubes, depending on the kind of medium used. Thus the activity of each drug was measured on our scale of concentra-



tions in terms of the smallest concentration required to inhibit the growth of an inoculum of about 1000 bacteria per cubic centimeter under conditions which, in the absence of drug, permitted heavy growth. This end-point was expressed as the "Minimal Inhibitory Concentration" in milligram per cent.

Repeated titrations of any one drug under the same test conditions usually resulted in the same end-point, although occasionally a shift to the next tube, above or below, was noted. In simultaneous titrations of the four drugs a shift in end-point for one was nearly always accompanied by a similar shift for the others. Thus, while the value for the inhibitory end-point of any one compound could be reproduced within plus 100 and minus 50 per cent, our activity ratios are probably accurate to within plus 50 and minus 25 per cent (i.e., the accuracy of reading our dilution scale).

Titration of drug activity was carried out in six kinds of test medium. The basic medium was the synthetic mixture of asparagine, glucose, ammonium sulfate and other salts described by Sahyun and his associates (26). The other test media consisted of the basic medium plus: (1) 200 mgm. per cent of an acid hydrolysate of casein (Casamino acids—Difco); (2) 200 mgm. per cent of an acid hydrolysate of gluten (Glutamino acids—Difco); (3) 200 mgm. per cent Tryptose peptone; (4) 2 mgm. per cent *d*-l-methionine; (5) various concentrations of *p*-aminobenzoic acid

### Results

The activity of the four drugs was titrated simultaneously against the Ward strain of *E. coli* in the basic synthetic medium alone and in this medium with each of the following additions: 200 mgm. per cent Casamino acids; 200 mgm. per cent Tryptose peptone; 10, 1, 0.1, 0.01 and 0.001 mgm. per cent *p*-aminobenzoic acid. Typical end-points (expressed as minimal inhibitory concentrations in milligrams per cent  $\times 100$ ) and activity ratios based on sulfathiazole are given in table 7. In the basic synthetic medium, the activity ratios for sulfathiazole, sulfadiazine, sulfapyridine and sulfanilamide were: 1; 1; 1/4; and 1/64 respectively.<sup>6</sup> In all of the other test media the *absolute* activity of each drug decreased, in the sense that more drug was required to produce the same degree of inhibition (i.e., the end-point, or minimal inhibitory concentration increased). However, the *relative* activity of sulfathiazole, sulfadiazine and sulfapyridine remained constant in all media tested. Sulfanilamide, on the other hand, appeared to be *relatively* more active in the media containing either peptone or the higher concentrations of *p*-aminobenzoic acid.

Determinations of activity against the MacLeod strain were carried out in the basic medium, alone and with the following additions: 200 mgm. per cent Casamino acids; 200 mgm. per cent Glutamino acids; 2 mgm. per cent methionine; 2 mgm. per cent *p*-aminobenzoic acid, and 200 mgm. per cent Tryptose peptone. Results are given in table 8.

<sup>6</sup> We wish to thank the Difco Laboratories for the Casamino and Glutamino Acids used in this work.

<sup>6</sup> Activity ratios have been based on sulfathiazole as the reference standard in each case. This was done merely for convenience as the same conclusions would have been reached had any one of the other drugs been chosen as the reference standard.



The MacLeod strain of *E. coli* appeared to be more susceptible than the Ward strain to the action of each drug. This is shown by a comparison of the end-points obtained whenever the same kind of test medium was used

TABLE 7

*Activity end-points and ratios with the Ward strain of E. coli in vitro*

SYNTHETIC MEDIUM PLUS:	NUMBER OF TESTS*	MINIMAL INHIBITORY CONCENTRATION IN MG. PER CENT $\times 100$			
		Sulfa-thiazole	Sulfa-diazine	Sulfapyridine	Sulfanilamide
.....	12	2 <sup>4</sup> (1)	2 <sup>4</sup> (1)	2 <sup>6</sup> (1/4)	2 <sup>10</sup> (1/64)
Casamino acids, 200 mgm. per cent.....	3	2 <sup>7</sup> (1)	2 <sup>7</sup> (1)	2 <sup>9</sup> (1/4)	2 <sup>13</sup> (1/64)
<i>p</i> -Aminobenzoic acid:					
0.001 mgm. per cent.....	1	2 <sup>5</sup> (1)		2 <sup>7</sup> (1/4)	2 <sup>11</sup> (1/64)
0.01 mgm. per cent.....	1	2 <sup>7</sup> (1)		2 <sup>9</sup> (1/4)	2 <sup>13</sup> (1/64)
0.1 mgm. per cent.....	1	2 <sup>10</sup> (1)		2 <sup>12</sup> (1/4)	2 <sup>15</sup> (1/32)
1 mgm. per cent.....	1	2 <sup>12</sup> (1)		2 <sup>14</sup> (1/4)	2 <sup>16</sup> (1/16)
10 mgm. per cent.....	2	2 <sup>13</sup> (1)		>2 <sup>14</sup> (1/4)†	2 <sup>16</sup> (1/8)
Tryptose peptone, 200 mgm. per cent.....	2	2 <sup>12</sup> (1)	2 <sup>12</sup> (1)	2 <sup>14</sup> (1/4)	2 <sup>15</sup> (1/8)

First figure in each column is the Minimal Inhibitory Concentration in milligrams per cent  $\times 100$ , e.g., 2<sup>4</sup> represents 0.16 mgm. per cent.

Figures in parentheses represent activity ratios of the drugs based on sulfathiazole.

\* Number of tests on which activity ratios are based.

† Estimated.

TABLE 8

*Activity end-points and ratios with the MacLeod strain of E. coli in vitro*

SYNTHETIC MEDIUM PLUS:	NUMBER OF TESTS*	MINIMAL INHIBITORY CONCENTRATION IN MG. PER CENT $\times 100$			
		Sulfa-thiazole	Sulfa-diazine	Sulfapyridine	Sulfanilamide
.....	5	2 <sup>1</sup> (1)	2 <sup>1</sup> (1)	2 <sup>4</sup> (1/8)	2 <sup>8</sup> (1/128)
Casamino acids, 200 mgm. per cent.....	3	2 <sup>5</sup> (1)	2 <sup>5</sup> (1)	2 <sup>8</sup> (1/8)	2 <sup>12</sup> (1/128)
Glutamino acids, 200 mgm. per cent.....	1	2 <sup>5</sup> (1)	2 <sup>6</sup> (1)	2 <sup>9</sup> (1/8)	2 <sup>13</sup> (1/128)
Methionine, 2 mgm. per cent..	1	2 <sup>3</sup> (1)	2 <sup>5</sup> (1)	2 <sup>7</sup> (1/4)	2 <sup>11</sup> (1/64)
<i>p</i> -Aminobenzoic acid, 2 mgm. per cent.....	1	2 <sup>12</sup> (1)	2 <sup>12</sup> (1)	2 <sup>14</sup> (1/4)	2 <sup>16</sup> (1/16)
Tryptose peptone, 200 mgm. per cent.....	2	2 <sup>11</sup> (1)	2 <sup>12</sup> (1/2)	2 <sup>14</sup> (1/8)	2 <sup>15</sup> (1/16)

First figure in each column is the Minimal Inhibitory Concentration in milligrams per cent  $\times 100$ , e.g., 2<sup>1</sup> represents 0.02 mgm. per cent.

Figures in parentheses represent activity ratios of the drugs based on sulfathiazole.

\* Number of tests on which activity ratios are based.



for both strains (tables 7 and 8). As with the Ward strain, the addition of various substances to the basic synthetic medium again caused a decrease in the *absolute* activity of each drug without, however, affecting the *relative* activity of sulfathiazole, sulfadiazine and sulfapyridine to a very marked extent. Here, again, sulfanilamide was an exception in that its *relative* activity appeared to increase in the presence of either peptone or *p*-aminobenzoic acid.

Titration of drug activity against the Klinefelter strain were not carried out in the plain synthetic medium because of erratic growth in the control tubes with the routine inoculum. However, suitable test conditions for this strain were obtained by the addition of 200 mgm. per cent of Casamino acids,

TABLE 9

*Activity end-points and ratios with the Klinefelter strain of E. coli in vitro*

SYNTHETIC MEDIUM PLUS:	NUMBER OF TESTS*	MINIMAL INHIBITORY CONCENTRATION IN MGM. PER CENT $\times$ 100			
		Sulfa- thiazole	Sulfa- diazine	Sulfapyridine	Sulfanilamide
Casamino acids, 200 mgm. per cent. . . . .	2	2 <sup>1</sup> (1)	2 <sup>1</sup> (1)	2 <sup>10</sup> (1/4)	2 <sup>14</sup> (1/64)
Glutamino acids, 200 mgm. per cent. . . . .	2	2 <sup>7</sup> (1)	2 <sup>7</sup> (1)	2 <sup>10</sup> (1/8)	2 <sup>14</sup> (1/128)
Methionine, 2 mgm. per cent . . . . .	2	2 <sup>7</sup> (1)	2 <sup>7</sup> (1)	2 <sup>9</sup> (1/4)	2 <sup>13</sup> (1/64)
<i>p</i> -Aminobenzoic acid, 2 mgm. per cent. . . . .	2	2 <sup>11</sup> (1)	2 <sup>11</sup> (1)	2 <sup>14</sup> (1/8)	2 <sup>15</sup> (1/16)
Tryptose peptone, 200 mgm. per cent. . . . .	2	2 <sup>12</sup> (1)	2 <sup>12</sup> (1)	2 <sup>14</sup> (1/4)	2 <sup>15</sup> (1/8)

First figure in each column is the Minimal Inhibitory Concentration in milligrams per cent  $\times$  100, e.g., 2<sup>1</sup> represents 2.56 mgm. per cent.

Figures in parentheses represent activity ratios of the drugs based on sulfathiazole.

\* Number of tests on which activity ratios are based.

Glutamino acids or Tryptose peptone, or 2 mgm. per cent of either methionine or *p*-aminobenzoic acid to the basic synthetic medium. The results are given in table 9. From the standpoint of activity ratios, these results agree (within experimental error) with the data on the Ward and MacLeod strains.

#### DISCUSSION

The inadequacy of the drug-diet method of therapy in a colon bacillus infection as opposed to its efficacy in streptococcus and pneumococcus infections in mice can be explained by the failure of the mice to ingest more than traces of the drug during the first twelve hours after infection. This was due to the toxic effect of the mucin which was used to enhance the virulence of the bacteria. Since duration of the blood concentration for only



14 to 16 hours is sufficient for optimal therapeutic effect, the drug-diet method is unsatisfactory for treatment of this particular infection. The occasional survival obtained with the drug-diet method appears to depend on the amount of drug in the animal at the time of infection, and on the rate at which the particular drug is eliminated.

In the case of streptococcus and pneumococcus infections, where no mucin is used, the majority of mice eat quite normally after infection. In addition, the duration of blood concentration for optimal therapeutic effect is between 3 and 6 days as opposed to less than 1 day in the case of the colon bacillus infection.

Due to the fact that optimal results are attained in the case of the latter infection with 14- to 16-hour therapy, it is practicable to maintain a more or less constant blood concentration of drugs by a suitable *per os* dosage schedule. The experimental basis for the quantitative comparison of drugs in the present study was established in experiments with sulfathiazole. In most of our experiments the duration of the maintained blood concentration was 10 to 12 hours instead of the optimal period of 14 to 16 hours. Previous experience with streptococcus and pneumococcus infections led us to believe that this would not affect seriously the results of our comparison. It was proven (table 6) that the activity ratio of sulfanilamide to sulfathiazole was the same (within experimental error) for 14- to 16-hour therapy as it was for 10- to 12-hour therapy.

The fluctuations in the blood concentration-time curves (fig. 1) vary with the different drugs. There is no way of assessing the importance of these fluctuations but we do not believe that they seriously influence the results. The estimation of the average blood concentrations during therapy by inspection of blood concentration-time curves obtained in preliminary experiments appears to be of sufficient accuracy considering the nature of the data.

If sulfanilamide is taken as the standard of comparison (1.0) instead of sulfathiazole, the activity ratios with their standard errors based on the S.B.C.<sub>50</sub> in milligrams per cent of sulfapyridine, sulfathiazole and sulfadiazine are:  $6.3 \pm 0.5$ ;  $10.3 \pm 1.0$ ; and  $11.2 \pm 1.2$  respectively. This shows that sulfathiazole and sulfadiazine are of equal activity (within experimental error) and that both of these drugs are significantly more active than sulfapyridine, which in turn is significantly more active than sulfanilamide. Thus, the quantitative data obtained for the present infection as well as data previously obtained for a streptococcus (8) and a pneumococcus (9) infection support the idea of specificity on the part of these drugs for different bacterial infections in mice.

By the use of a quantitative method for comparing the antibacterial activity of the four drugs in various media we have obtained results (tables 7, 8 and 9) which seem to justify the following conclusions. (1) Absolute values for drug activity were markedly influenced by the composition of the test



medium. (2) The relative activities (ratios) of sulfathiazole, sulfadiazine and sulfapyridine remained constant, within experimental error, under various conditions obtained by adding known anti-drug factors or certain protein hydrolysates to a synthetic medium. (3) The relative activity of sulfanilamide appeared to be increased in the presence of peptone or *p*-aminobenzoic acid. (4) There appears to be at least a qualitative agreement between the *in vivo* and *in vitro* activity of these drugs.

One might conclude from the striking increase in the relative activity of sulfanilamide that the anti-drug effect of either *p*-aminobenzoic acid or some factor in peptone is exerted to a much less extent on this drug than on its heterocyclic derivatives. An alternative explanation is that sulfanilamide has a relatively greater inhibitory effect on the metabolism of *p*-aminobenzoic acid and peptone by the colon bacillus. However, one cannot discard the possibility that the phenomenon is an artefact due to a non-specific effect of the high end-point concentrations of sulfanilamide in these media. A further study of the *in vitro* activity of sulfanilamide derivatives is in progress.

#### SUMMARY

The drug-diet method was shown to be unsatisfactory for treatment of an *E. coli* infection in mice. More or less constant blood concentrations could be maintained by administration of drugs *per os*, by tubing, according to suitable dosage schedules. The optimal duration of therapy for this infection was found to be 12 to 14 hours. The therapeutic activity of four drugs was determined on the basis of blood concentrations maintained for 10 to 12 hours or longer. On the basis of blood concentrations in milligrams per cent, sulfapyridine was 6 times, sulfathiazole 10 times and sulfadiazine 11 times as active as sulfanilamide *in vivo*. The estimated error of each of these activity ratios was  $\pm 10$  per cent. On the basis of minimal inhibitory concentration in milligrams per cent, sulfapyridine was 16 times, and sulfathiazole and sulfadiazine 64 times as active as sulfanilamide *in vitro*.

We wish to thank Miss Dorothea Babbitt for technical assistance in determination of blood concentrations.

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## POTENTIATION OF ACETYLCHOLINE BY ALCOHOL AND ETHER

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Received for publication January 23, 1941

Chang and Gaddum in 1933 (1) recommended extracting acetylcholine from tissues by macerating in trichloroacetic acid and removing the acid by shaking with ether. The ether was removed from the solution before assay, by concentration at low pressure and 40°C. When Chang and Wong (2) later extracted acetylcholine from the human placenta, they macerated the tissue in 95 per cent ethyl alcohol, filtered and assayed the crude alcoholic (about 70 per cent) extract directly. They assumed that the alcohol would not interfere with the assay by the frog rectus method, since they found that equivalent volumes of alcohol alone, added to the bath, caused no contraction of the muscle. Ettinger and Bateman showed, however, (3) that alcohol potentiates acetylcholine, and for that reason Chang's reported yields of acetylcholine from the placenta were about twice what they should be. Emmelin (4) has since shown that when various alcohols or certain other narcotics are added to a bath of Ringer's solution containing the frog rectus a short time before introducing acetylcholine, the contraction produced is greatly enhanced. He did not measure the potentiation. Emmelin found that these narcotics enhanced the contraction of the normal as well as of the eserized rectus, and so concluded that they do not act through inhibition of esterase activity.

The purpose of this paper is to relate the degree to which the effect of acetylcholine on the frog's rectus abdominis may be magnified, if the acetylcholine is dissolved in varying strengths of ethyl alcohol or diethyl ether, before being added to the bath containing the rectus. Potentiation is expressed in relation to concentration of alcohol in the solvent, rather than final concentration in the bath since (a) when tissues are extracted with alcohol, the concentration of alcohol in the extract is readily known, while the final concentration in the test bath will vary with the amount of the extract used for assay, and (b) the maximal potentiation was found proportional to the concentration of alcohol in the solution to be assayed, not to the final concentration of alcohol in the bath.

We have also investigated the effect of alcohol on the response of the cat's blood-pressure and the rabbit's intestine to acetylcholine.



## 1. ALCOHOLIC POTENTIATION ON THE FROG'S RECTUS

*A. Method*

The rectus abdominis of the frog (*R. pipiens*) was suspended in a Magnus bath of Ringer's solution, volume 21 cc. Oxygen was bubbled through the bath. The muscle was attached to a lever with 10 fold magnification and a tension of 3 grams. Solutions were introduced from a 1 cc. graduated syringe. The contraction produced by alcoholic solutions of acetylcholine (1:100,000 to 1:1,000,000) was matched against the contraction produced by solutions in Ringer's solution of the same concentration. Acetylcholine was allowed to produce contraction for three minutes and was then washed out. The muscle was allowed to rest 10 minutes between stimulations. To produce eserization of the rectus, 0.22 mg. of eserine sulphate was added to the bath and allowed to act for at least thirty minutes; then, after each washing, 0.22 mg. was added, after the method of Chang and Gaddum.

*B. Effect of alcohol alone*

Alcoholic solutions of strengths 30 to 95 per cent, without acetylcholine, were injected in amounts corresponding to the volumes of acetylcholine solutions normally used for assay, i.e., up to 0.5 cc., to see whether they caused contraction of the normal or eserinized rectus. This produced final concentrations of alcohol of 0.3 to 2.4 per cent. Contraction was produced in only 5 of 50 attempts. The contraction was not typical of the acetylcholine effect.

*C. Effect of alcoholic solutions of acetylcholine*

The alcoholic solutions of acetylcholine tested contained from 30 to 95 per cent alcohol and 1:100,000 or (rarely) 1:1,000,000 parts of acetylcholine. The volumes injected into the muscle-bath were from 0.05 to 0.4 cc., and the resulting dilution of the alcohol was 0.16 to 1.5 per cent depending on the dose and concentration. Emmelin reported that, when a frog rectus was suspended in a bath of Ringer to which ethyl alcohol was added before an injection of acetylcholine, the alcohol must have a minimum concentration of 1:1000, and must be allowed to act for at least thirty seconds before it could enhance the contraction produced by acetylcholine. He usually allowed the alcohol to act for three minutes before injecting the acetylcholine. We compared Emmelin's method, viz., allowing alcohol to act for one to three minutes on the rectus before introducing the aqueous acetylcholine, with our own, viz., introducing the alcoholic acetylcholine into the Ringer bath without any previous treatment. Usually we found the potentiation was greater by our method than by Emmelin's (fig. 1). Obviously it is not the final concentration of alcohol alone which determines the potentiation.

Potentiation was produced by alcohol on both normal and eserinized recti. It increased with increasing concentrations of alcohol in the solutions of acetylcholine injected into the bath, up to 70 per cent (see figs. 2 and 3) and fell off



thereafter. At the high level the potentiation on the normal rectus exceeded that on the eserized preparations. Fig. 4 shows the *maximum* potentiation demonstrated by various concentrations of alcohol in more than fifty experiments. The *mean* potentiation for the various strengths of alcohol falls below, but parallel to this curve. Thus while the maximum potentiations on the normal and eserized rectus were 6 and 3 respectively, produced by 70 per cent alcohol, the mean potentiations produced by that concentration of alcohol were 4 and 2 respectively.

While the potentiation increased with increasing concentration of alcohol in the injected solution, it was not proportional to the resulting concentration of alcohol in the Magnus bath. In two experiments in which acetylcholine in 70 per cent alcohol was potentiated four times, the volumes injected (0.05 and

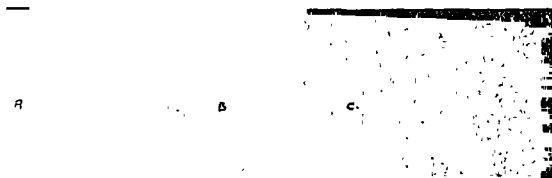


FIG. 1. COMPARISON OF RESPONSE OF NORMAL RECTUS ABDOMINIS TO (A) ALCOHOLIC ACETYLCHOLINE, (B) AQUEOUS ACETYLCHOLINE, (C) AQUEOUS ACETYLCHOLINE AFTER TREATMENT WITH ALCOHOL FOR ONE MINUTE

The final  
titration was

0.4 cc.) were such that the resulting dilutions of alcohol were 0.16 and 1.33 per cent respectively.

On the eserized rectus in three experiments the solutions assayed contained respectively 30, 70 and 95 per cent alcohol, the potentiations were 1.25, 3, and 1.3, yet the final concentrations of alcohol were respectively 0.57, 0.33 and 1.25 per cent.

## 2. POTENTIATION OF ACETYLCHOLINE ON THE FROG'S RECTUS BY ETHER

In order to determine the potentiation produced by ether, solutions of acetylcholine, 1:100,000 and 1:1,000,000, were made in Ringer's solution, saturated and half-saturated with ether. These were compared with standard aqueous solutions as in the experiments with alcohol.

Ringer's solution, saturated or half-saturated with ether, produced no contraction of the rectus in amounts up to 0.5 cc. Acetylcholine was potentiated by half-saturation with ether, 1.6 on the normal rectus, 1.1 on the eserized



A.

B.

C.

D.

FIG. 2. POTENTIATION OF ACETYLCHOLINE BY 30 PER CENT ALCOHOL ON NORMAL AND ESERINIZED RECTUS

4 $\gamma$  of acetylcholine in 30 per cent alcohol (A) produced contraction of the *normal rectus* equivalent to that produced by 5 $\gamma$  of acetylcholine in Ringer's solution (B). The potentiation was 1.25; the final alcoholic concentration, 0.57 per cent.

0.6 $\gamma$  of acetylcholine in 30 per cent alcohol (C) produced contraction of the *eserinized rectus* equivalent to that produced by 1 $\gamma$  of acetylcholine in Ringer (D). The potentiation was 1.66; the final concentration of alcohol, 0.9 per cent.

rectus. Full saturation with ether produced a potentiation of 2.25 on the normal rectus, and 2.0 on the eserinated rectus.







if the eserization did not produce total suppression of the esterase. In the standard method of Chang and Gaddum this is the case. Their method of

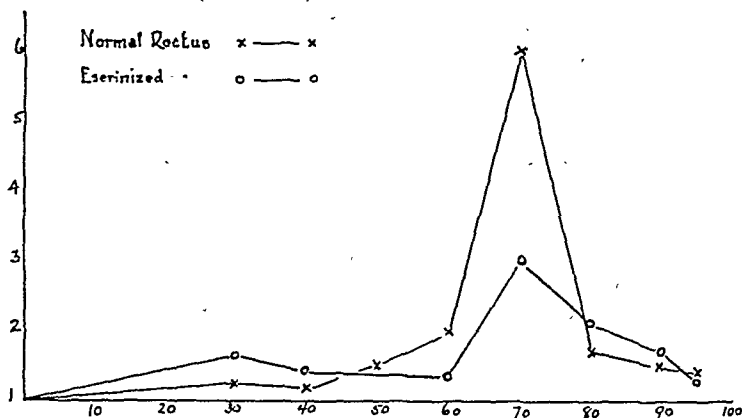


FIG. 4. MAXIMUM POTENTIATION OF ACETYLCHOLINE ON THE RECTUS ABDOMINIS OF THE FROG PRODUCED BY ETHYL ALCOHOL

Ordinate:  $\gamma$  equivalent of aqueous acetylcholine per  $1\gamma$  of alcoholic solution.

Abscissa: per cent of alcohol in the solutions of acetylcholine used for assay (not final alcoholic concentration in the Magnus bath).

The points on these curves show the *maximum* potentiation produced on the normal and eserinated rectus in more than 50 experiments, when acetylcholine, dissolved in alcohol, was injected in volumes of 0.05 cc. to 0.4 cc. into a muscle-bath of 21 cc. volume. While the maximum level was not always reached, the curves indicate the *possible* error in an assay of an acetylcholine solution containing alcohol.

A.

B.

FIG. 5. FROG RECTUS, ESERINIZED, 1ST AND 2ND APPLICATIONS OF ACETYLCHOLINE

Acetylcholine after prolonged eserization (A—90') produces greater contraction than after washing and brief eserization (B—7').

producing "nearly maximal sensitization to acetylcholine" is (a) the addition of eserine in quantity sufficient to give a concentration of  $10^{-3}$ , and allowing this



to act for *thirty* minutes before testing the muscle, and (b) the addition of a similar amount of eserine after each washing, and allowing this to act seven minutes before the next assay. When this practice is followed, consistent results are produced with aqueous solutions of acetylcholine. When, however, the first eserization was allowed to proceed for an hour or *ninety* minutes before the muscle was tested, we found the first response to acetylcholine much greater than the responses after washing and later eserization (fig. 5). Obviously *prolonged* eserization produced greater anti-esterase effect. The anti-esterase effect of eserine may apparently be also supplemented by alcohol.

Alcohol might also potentiate acetylcholine by making the rectus more permeable.

### 3. EFFECTS OF ALCOHOLIC AND ETHEREAL ACETYLCHOLINE ON THE RABBIT'S INTESTINE

Solutions of acetylcholine in 70 and 80 per cent alcohol, and solutions saturated and half-saturated with ether, were tested on the rabbit's intestine. Injected into a bath of 40 cc. volume, in amounts from 0.2 to 0.5 cc. they produced less contraction than equivalent amounts of aqueous acetylcholine. Rydin (5) has reported a similar effect with ether, and Guillot and Ong Sian Gwan (6) found that ethyl alcohol decreased the action of acetylcholine on the intestine of the guinea-pig.

### 4. EFFECT OF ALCOHOLIC ACETYLCHOLINE ON THE CAT'S BLOOD-PRESSURE

The depressor effect of acetylcholine in 50 and 70 per cent alcohol was tested on the cat under dial. In volumes up to 0.2 cc., the depressor effect was equal to that produced by equivalent amounts of aqueous acetylcholine; volumes of 0.3 cc. produced a greater fall of blood-pressure.

### CONCLUSIONS

Solutions of acetylcholine in ethyl alcohol and diethyl ether cause greater contraction of the frog rectus than do aqueous solutions. The potentiation is greater on the normal than on the eserized rectus. The maximal potentiation is produced by alcohol in concentrations of 60 to 80 per cent, viz., as much as six times on the normal rectus and three times on the eserized rectus. The potentiation probably depends, at least in part, on an anti-esterase effect.

Solutions of acetylcholine in alcohol or ether cause less stimulation of the rabbit's intestine than do aqueous solutions. Alcohol in a solution of acetylcholine may also potentiate the depressor effect on the cat.

Tissue extracts should be freed of alcohol or ether before being assayed by any of the three methods described.



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# STUDIES CONCERNING THE ANESTHETIC ACTION OF STEROID HORMONES

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Received for publication June 13, 1941

It has recently been found (1-3) that steroid hormones, such as progesterone, desoxycorticosterone acetate (D.C.A.), androgens and estrogens, cause general anesthesia in various experimental animals (rat, mouse, guinea pig, rabbit and cat) if these compounds are administered intraperitoneally or intravenously. Anesthesia cannot be obtained even with huge doses if the same hormones are administered subcutaneously. Hence it would appear that in order to obtain the anesthetic effect the organism must suddenly be flooded with comparatively large amounts of these compounds and that even the largest doses given subcutaneously do not result in a sufficiently high hormone concentration in blood and tissues to elicit this response. Comparative studies with hormonally inactive steroids revealed that only compounds having a hormone action produce anesthesia even though the anesthetic effect is not dependent upon any particular type of hormone activity. It is true that progesterone and D.C.A. are much more potent anesthetics than the androgens, which in turn are more potent than the estrogens, but all these compounds possess some measure of anesthetic action while even their closest chemical derivatives if hormonally inactive are devoid of it. It is of particular interest, for instance that D.C.A. and progesterone which are the most active anesthetics among the steroids so far examined, differ from  $\Delta^4$ -cholestenone only in their  $C_{17}$  side chain yet the latter compound which has no hormonal effect, is entirely free of anesthetic action. It would be suggestive to assume that in compounds having the steroid nucleus of these  $\Delta^4$ -3 ketones the structure of the side chain at  $C_{17}$  determines the anesthetic action. However testosterone—an active androgen—in which the chemical configuration of the steroid nucleus is exactly the same as in the above mentioned three compounds, is a potent anesthetic although it has no  $C_{17}$  side chain at all. These observations led to the formulation of what appears to be the *fundamental law of steroid hormone anesthesia*, namely that *all compounds having a steroid hormone action are capable of producing anesthesia while no compound devoid of hormone action possesses this power* (1-3). It has also been observed that the anesthetic effect of all steroid hormones so far examined is more marked in female than in male animals. Spaying does not alter the sensi-



tivity of females but castration raises the responsiveness of males to the female level. Chronic subcutaneous administration of comparatively small quantities of androgens, on the other hand, diminishes the sensitivity of females and castrate males to the level characteristic of intact males (1, 4). The anesthetic effect of the steroid hormones is greatly increased after partial hepatectomy in both sexes and it appears probable that their detoxification occurs in the liver (2). Following pretreatment with intraperitoneal injections of a certain steroid hormone, the sensitivity of the organism diminishes, especially to the anesthetic effect of this same hormone, but also though less markedly to that of other hormonally active steroids (3).

In the present communication, experiments on rats will be reported which were designed to test the validity of the above mentioned fundamental law of steroid hormone anesthesia with a series of compounds which have not been studied so far from this point of view. Other experiments to be reported here are concerned with the conditions influencing the course of hormone anesthesia.

#### THE ANESTHETIC ACTION OF CERTAIN COMPOUNDS WHICH HAVE HITHERTO NOT BEEN TESTED FOR SUCH AN EFFECT

In our first series of experiments, we tested two adrenal steroids which up to the present have not been examined for a possible anesthetic effect. These were Reichstein's compound "J" (17-ethylandrostan-3,17,20-triol) which was used in the form of its 3,20-diacetate, and Kendall's compound "A" (17-ethyl- $\Delta^4$ -androstene-21-ol-3,11,20-trione or dehydrocorticosterone). In the same series, we also tested the possible anesthetic action of ascorbic acid since recently Israel and Meranze (5) claimed that this compound possesses progesterone-like actions and progesterone is a very potent anesthetic. "J"-diacetate was administered in peanut oil containing 25 mg. per cc., Kendall's "A" also in peanut oil in the same concentration, and ascorbic acid in aqueous solution containing 50 mg. per cc. All compounds were administered intraperitoneally. Table 1 gives the average of the maximum anesthesia observed in each group. The slightest degree of an anesthetic effect was marked +, medium anesthesia ++, and deep anesthesia with abolition of the righting reflex +++.

It is of particular interest to note that Reichstein's "J"-diacetate proved inactive in doses up to 30 mg. although in animals of this size, 4 mg. of D.C.A. suffices to produce marked anesthesia. It will be remembered that Reichstein (6) found "J"-diacetate to be completely inactive as an adrenal cortical hormone in doses up to 0.5 mg. in the Everse-de Fremery test in which, according to Reichstein and v. Euw (7), 0.04 mg. of D.C.A. suffice to give a 50 per cent positive result. It is seen that although "J" is a steroid naturally occurring in the adrenal cortex, it has no cortical hormone activity and accordingly, no anesthetic action. This observation supports the law according



to which hormonally inactive steroids are devoid of anesthetic activity and shows that this is true even of hormonally inactive steroids which occur in nature as constituents of endocrine glands.

Our next experiments were concerned with the action of acetoxypregnenolone (21-acetate of 17-ethyl- $\Delta^4$ -androstene-3,21-diol-20-one), a compound which differs radically from all known adrenal cortical steroids in that it possesses a double bond in position  $C_{5-6}$ . Probably because of this structure, it has never been tested for any possible physiological activity although it is

TABLE 1

*Test of the anesthetic effect of Reichstein's compound "J"-diacetate, Kendall's compound "A" and ascorbic acid*

TREATMENT	NUMBER OF ANIMALS		AVERAGE BODY WEIGHT	AVERAGE DEPTH OF ANESTHESIA
	Males	Females		
			grams	
Reichstein's "J"-diacetate:				
5 mg.....	1		53	0
10 mg.....	1		56	0
15 mg.....		1	60	0
20 mg.....		1	61	0
30 mg.....	1		40	0
Kendall's "A":				
2 mg.....	3		40	trace
3 mg.....	3		57	0
5 mg.....		3	54	0
10 mg.....		2	44	+++
20 mg.....		1	54	+++
Ascorbic acid:				
15 mg.....	5		45	0
25 mg.....	5		48	0
50 mg.....		5	47	0
100 mg.....		5	53	0

readily available by partial synthesis as it represents an intermediary step in the commercial manufacture of D.C.A. Most surprisingly intraperitoneal administration of this compound revealed it to have a definite anesthetic power and at first it was thought that this observation invalidates the fundamental law of steroid hormone action as it was tacitly assumed that acetoxypregnenolone is not hormonally active. However, encouraged by the discovery of its anesthetic action, the compound has subsequently been tested for other physiological effects and was found to possess definite adrenal cortical hormone activity. Highly purified samples of this substance maintain the life of adrenalectomized rats (weighing 35-40 grams) in doses of 200 micrograms



per day. The substance also depresses the blood chlorides in the intact rat as D.C.A. does, although it is quantitatively less active in this respect. The first sample we had at our disposal was not quite pure having a M.P. of 175-178° and thus its activity might have been due to the presence of some contamination. Yet subsequent assays showed that a thoroughly purified sample having the correct M.P. of 183-184° was also active. The detailed results of our pharmacological assays concerning the adrenal cortical hormone activity of acetoxypregnenolone will be published elsewhere together with studies concerning its possible progestational, estrogenic and androgenic activity. Table 2 summarizes the results of our studies concerning its anesthetic effect. In all cases the compound was administered in peanut oil containing 20 mg. per cc.

TABLE 2  
*Anesthetic effect of acetoxypregnenolone (21-acetate of  
17-ethyl- $\Delta^5$ -androstene-3,21-diol-20-one)*

TREATMENT	NUMBER OF ANIMALS		AVERAGE BODY WEIGHT	AVERAGE DEPTH OF ANESTHESIA
	Males	Females		
			grams	
Acetoxypregnenolone, M.P. 175-178°:				
5 mg.....	1	6	79	+
10 mg.....	2	2	40	+
15 mg.....		4	41	++
20 mg.....		6	51	+++
25 mg.....	4		52	+++
Acetoxypregnenolone, M.P. 183-184°:				
10 mg.....		2	37	+
20 mg.....		2	37	+++

The data in table 2 clearly indicate that acetoxypregnenolone possesses the power of producing anesthesia, and in the case of this compound the fundamental law of steroid anesthesia proved of heuristic value since it was the discovery of its anesthetic action that led to pharmacological studies which established its hormonal activity. Thus in this case of a compound not known to occur naturally in the animal body, the rule still applies that anesthetic steroids are hormonally active and *vice versa*. Since the number of known steroid hormone derivatives is enormous and continues to increase the biological assay of these preparations lags far behind their preparation by the chemists. Unless further research should reveal important exceptions to the fundamental law of steroid hormone anesthesia, it would appear that a test of anesthetic potency would represent a valuable short cut in the detection of hormonal activity. A single test, necessitating only a few milligrams of the steroid and a few hours time, is likely to detect those compounds which deserve



a more detailed pharmacological analysis of their possible progestational androgenic, estrogenic and adrenal cortical activity.

Although ascorbic acid proved to have no anesthetic action, it appeared of some importance to establish whether other vitamins are active in this respect. Especially those related to sex hormones, e.g. vitamin E, and those having structural similarities to the steroid hormones, e.g. calciferol, appeared of interest in this connection. We shall not tabulate the results of these investigations in detail, suffice it to say that in female rats weighing 45 grams on the average, calciferol in doses up to 30 mg. dissolved in 0.05 cc. of peanut oil and *dl*- $\alpha$ -tocopherol acetate an oily substance which was injected as such in doses up to 750 mg. (!) proved entirely devoid of anesthetic activity. These observations indicate that vitamin activity does not endow a chemical compound with the power of producing anesthesia in the manner in which hormonal activity does.

TABLE 3

*Anesthetic effect of combined treatment with progesterone and desoxycorticosterone acetate*

TREATMENT	NUMBER OF ANIMALS		AVERAGE BODY WEIGHT	AVERAGE DEPTH OF ANESTHESIA
	Males	Females		
Progesterone, 7 mg.. .. . . .	5	5	70	+++
Desoxycorticosterone acetate, 7 mg. . .	5	5	71	+
Desoxycorticosterone acetate, 3.5 mg. and progesterone, 3.5 mg.....	5	5	72	++

THE RESULT OF COMBINED TREATMENT WITH TWO ANESTHETIC STEROIDS

Since in many instances simultaneous administration of two anesthetic drugs elicits a more pronounced anesthesia than could be expected by mere summation of their effect, it appeared of interest to establish whether two anesthetic steroids, for instance D.C.A. and progesterone, would produce a particularly marked effect on the nervous system. In all groups recorded in table 3, the steroids were administered intraperitoneally in a solution containing 40 mg. per cc. of peanut oil. The solution given to the group receiving the combination treatment consequently contained 20 mg. of progesterone and 20 mg. of D.C.A. per cc. in the same solvent.

Our results indicate that there is no potentiation of the anesthetic effect of these two steroids. It is noteworthy that in these tests progesterone proved definitely more potent than D.C.A. while in previous experiments (1), the latter was found to be more active. The explanation of this apparent discrepancy is that our earlier experiments were performed on larger rats than those used in the present investigation. It is evident from all the work performed in this laboratory on the anesthetic effect of the above steroids that



in animals heavier than about 80 grams D.C.A. produces more marked anesthesia than progesterone, this difference becoming more and more evident as the weight of the animal rises. On the other hand, small rats weighing 80 grams or less are definitely more sensitive to progesterone.

EFFECT OF HYPOPHYSECTOMY, ADRENALECTOMY, THYROIDECTOMY AND  
NEPHRECTOMY ON THE COURSE OF STEROID HORMONE ANESTHESIA

In the opening section of this communication we mentioned previous experiments which showed that partial hepatectomy greatly increases the anesthetic effect of steroid hormones and that our observations support the view according to which the liver plays an important rôle in the detoxification of these compounds. On the other hand, it is well known that the liver is, in many respects, under the regulating influence of the adrenal cortex. This has first been shown in experiments demonstrating that fat deposition in liver cells is inhibited by adrenalectomy unless adrenal cortical hormone therapy is given (8, 9). It appeared of interest therefore to determine whether adrenalectomy, or functional inactivation of the adrenal cortex by hypophysectomy, would influence the course of steroid hormone anesthesia. It appeared important furthermore to establish the possible effect of a decrease in the B.M.R. caused by thyroidectomy. As it is well known that a large part of the various steroid hormones is eliminated in a conjugated form through the kidneys it was also deemed advisable to determine whether total nephrectomy would prolong the course of steroid hormone anesthesia. The adrenalectomies in group I and the nephrectomies in group III were performed two hours before the test. The operations in group II were performed four days, in group III, seven days, in groups IV and V, four days before the test. All steroids were administered intraperitoneally in peanut oil. The progesterone solution in groups I and II contained 30 mg. per cc., the D.C.A. in group III 40 mg. per cc., the testosterone in group IV 40 mg. per cc. and the  $\alpha$ -estradiol in group V 40 mg. per cc. As in all our experiments concerning the anesthetic action of these hormones, the steroids were dissolved in the peanut oil by heating and were injected in super-saturated solutions as soon as the oil reached body temperature. At this time fine crystals formed in the case of the more insoluble preparations such as  $\alpha$ -estradiol and it is not known how rapidly these crystals can be absorbed. It appears quite possible, however, that the relative inefficacy of the estrogens to produce anesthesia in these acute experiments is at least in part due to their comparative insolubility. It may incidentally be mentioned that relatively insoluble steroids (Kendall's "A", acetoxypregnenolone,  $\alpha$ -estradiol) tend to produce particularly prolonged anesthesia which may last 12-18 hrs. even if it is not very deep at any time. This may also be due to comparatively slow absorption. The readily soluble compounds such as progesterone tend to produce an anesthesia of great depth but comparatively short (1-2 hrs.) duration.



The experiments summarized in table 4 clearly indicate that adrenalectomy and hypophysectomy increase the anesthetic effect of various steroids while nephrectomy and thyroidectomy have no such effect. It is particularly noteworthy, however, that within the first few hours after adrenalectomy the animals are not more sensitive than intact controls and that their sensitivity develops only at a later period. This indicates that the actual presence of the

TABLE 4

*Effect of hypophysectomy, adrenalectomy, thyroidectomy and nephrectomy on the course of steroid hormone anesthesia*

GROUP		NUMBER OF ANIMALS		AVERAGE BODY WEIGHT	AVERAGE DEPTH OF ANESTHESIA
		Males	Females		
				grams	
I	Intact; progesterone, 4 mg.	5	5	41	++
	Adrenalectomized; progesterone, 4 mg.	10	10	40	++
II	Intact; progesterone, 3 mg.	5	5	39	+
	Hypophysectomized; progesterone, 3 mg.	5	5	41	+++
	Thyroidectomized; progesterone, 3 mg.	5	5	39	+
	Adrenalectomized; progesterone, 3 mg.	5	5	40	+++
III	Intact; desoxycorticosterone acetate, 5 mg.	5	5	60	+
	Hypophysectomized; desoxycorticosterone acetate, 5 mg.	5	5	55	+++
	Thyroidectomized; desoxycorticosterone acetate, 5 mg.	5	5	59	+
	Adrenalectomized; desoxycorticosterone acetate, 5 mg.	5	5	61	+++
	Nephrectomized; desoxycorticosterone acetate, 5 mg.	5	5	58	+
IV	Intact; testosterone, 10 mg.	5	5	50	0
	Adrenalectomized; testosterone, 10 mg.	5	5	52	++
V	Intact; estradiol, 30 mg.	5	5	55	0
	Adrenalectomized; estradiol, 30 mg.	5	5	53	+

adrenal cortex during the period of anesthesia is not the important factor, but that as adrenal insufficiency develops the sensitivity of the organism to this effect of the steroids gradually increases. Actually the difference in the degree of anesthesia produced by a hormone in the normal animal, on one hand, and the chronically adrenal or pituitary deficient rat on the other, was even more marked than can be shown by the crosses used in the table because the duration of the anesthesia was also greatly prolonged, not only its intensity.



It should be mentioned that this increase in sensitivity developed in the adrenalectomized animals although they were given 0.9 per cent NaCl instead of drinking water, immediately after the operation and were shifted to tap water only 24 hours before the test. It is especially noteworthy that the resistance of the animals decreases even towards D.C.A., that is, a physiological adrenal cortical steroid which prevents the onset of adrenal insufficiency in the suprarenalectomized rat. In adrenalectomized animals receiving D.C.A. by the intraperitoneal route we are confronted with the apparent paradox of an animal simultaneously displaying deficiency in, and overdosage with adrenal cortical hormone. It appears possible that the mechanism of the life-maintaining effect exerted by comparatively small doses of D.C.A., when they are given chronically by the subcutaneous route, is essentially different from the anesthetic action produced by the sudden flooding of the organism after D.C.A. is injected intraperitoneally. Perhaps the changes due to chronic suprarenal insufficiency are of such a nature that they cannot be abolished by sudden overdosage with large amounts of a cortical hormone. These findings may possibly also explain the occurrence of certain endocrine diseases in which overdosage and deficiency symptoms apparently resulting from the "dysfunction" of the same gland are simultaneously in evidence. The fact that hypophysectomy also increases hormone sensitivity speaks strongly in favor of the view that it is deficiency in the cortical part of the adrenal which is mainly responsible for the change in responsiveness, since ablation of the pituitary causes atrophy of the adrenal cortex without significantly influencing the medulla. The observation that thyroidectomy is ineffective is strong evidence against the view that hypophysectomy acts merely by decreasing the B.M.R. Finally the ineffectiveness of complete nephrectomy clearly indicates that the elimination of the injected hormone by way of the kidneys plays no significant rôle in the mechanism which helps the organism to combat the anesthetic effect of steroids.

Since it is well known that the resistance of adrenalectomized animals is decreased with regard to the toxic actions of a great many drugs, it seemed of importance to establish whether the sensitization to the anesthetic effect of the steroid hormones is a specific result of adrenal insufficiency. In order to answer this question, experiments were performed in which the anesthetic effect of chloroform, magnesium chloride and ether were studied comparatively in the intact and adrenalectomized animal. For this purpose 48 female rats having an average body weight of 62 grams (range 54-65 grams) were divided into six groups of eight animals each. Three groups were adrenalectomized while the other three served as controls. Three days after the adrenalectomy, one group of adrenalectomized and one group of control animals were simultaneously anesthetized with ether in the same large dessicator. They were removed from the jar when the anesthesia appeared to be complete. There was no difference either in the speed with which the anesthesia developed or in the recovery time in the two groups. The second experiment



was performed under identical conditions using chloroform as an anesthetic. Here again adrenalectomy did not influence the sensitivity of the animals in any way. The third group of eight intact and eight adrenalectomized rats were given a subcutaneous injection of 0.6 cc. of a 10 per cent magnesium chloride solution. This dose sufficed to induce deep anesthesia of approximately 30 minutes duration in all animals and it was observed that even in this case when the anesthetic was administered by injection, suprarenal insufficiency did not modify the sensitivity to its action. It is evident therefore that it is not merely because of the decrease in general resistance that adrenalectomy sensitizes to steroid anesthetics.

#### EFFECT OF ATROPINE, ACETYLCHOLINE AND VAGOTOMY ON STEROID HORMONE ANESTHESIA

In the course of our studies it was accidentally discovered that shortly after an atropine injection the resistance of animals towards the anesthetic effect of steroids increases. Because of the known parasympathetic action of atropine, this effect was studied in some detail and other experiments were designed to establish the influence of acetylcholine and vagotomy. The relevant experiments are summarized in table 5 in which the details of the experimental procedure are also given. It should be mentioned here, however, that atropine was administered in the form of its sulphate and acetylcholine in the form of its chloride both in aqueous solution by the subcutaneous route. The amount of progesterone indicated in the table was in all instances administered in 0.1 cc. of peanut oil intraperitoneally.

Our data clearly indicate that brief pretreatment with suitable doses of atropine invariably antagonized the anesthetic action of progesterone, while chronic pretreatment with the same compound had exactly the opposite effect. Cervical vagotomy had the same effect as chronic atropine treatment but since subdiaphragmatic vagotomy had only a very slight effect, if any, it remains doubtful whether the increase in sensitivity caused by cervical vagotomy is a specific result of this intervention or whether it is merely due to a decrease in resistance caused by the respiratory difficulties which are elicited by transection of the vagus above the origin of the recurrent laryngeal nerve. In any case acetylcholine did not influence progesterone anesthesia in any way and hence it is felt that at this time it is best simply to report these observations and to refrain from any speculation concerning the rôle of the vagus in this phenomenon.

#### EFFECT OF ADAPTATION TO FORMALDEHYDE AND FORCED MUSCULAR EXERCISE ON STEROID HORMONE ANESTHESIA

It has been shown that while an organism acquires a conspicuous degree of adaptation to a certain agent with which it is chronically treated, it becomes less and less resistant to other agents (10, 11). Since it is well known that during chronic treatment with atropine rats develop a great deal of resistance



TABLE 5

*Effect of atropine, acetylcholine and vagotomy on steroid hormone anesthesia*

GROUP	PRETREATMENT	DURATION OF PRE-TREATMENT	TREATMENT	NUMBER OF ANIMALS		AVERAGE BODY WEIGHT	AVERAGE DEPTH OF ANESTHESIA	NUMBER OF DEATHS
				Males	Females			
I	1 × 1 cc. 2% atropine 3 × 0.25 cc. 2% atropine 9 × 0.25 cc. 2% atropine	*	Progesterone, 4 mg.		6	grams 45	+++	6
			Progesterone, 4 mg.		6	46	+++	6
			Progesterone, 4 mg.		6	44	++	0
		4 hrs.	Progesterone, 4 mg.		6	47	++	4
II	6 × 0.25 cc. 1.2% atropine 9 × 0.25 cc. 1.2% atropine	5 hrs.	Progesterone, 4 mg. Progesterone, 4 mg.	5	5	53 54	++ <+	0 0
		8 hrs.	Progesterone, 4 mg.	5	5	54	+	0
III	4 × 0.25 cc. 2% atropine 8 × 0.25 cc. 2% atropine	3 hrs.	Progesterone, 6 mg. Progesterone, 6 mg.		6 6	64 65	+++ ++	0 0
		7 hrs.	Progesterone, 6 mg.		6	64	<+	0
IV	2 × 0.25 cc. 6% atropine	1 hr.	Progesterone, 4 mg. Progesterone, 4 mg.		8 8	42 41	+++ +	1 0
V	2 × 0.5 cc. 1.5% atropine	4 hrs.	Progesterone, 5 mg. Progesterone, 5 mg.	5 5	5 5	55 56	+++ +	0 0
VI	Progesterone, 6 mg. Progesterone, 6 mg.	1 hr.	1 × 1 cc. 6% atropine		6 6	68 66	+++ +++	0 0
VII	2 × 0.5 cc. 1.5% atropine daily	5 days	Progesterone, 7 mg. Progesterone, 7 mg.	10 10	10 10	78 70	+ +++	0 11
VIII	2 × 0.5 cc. 1.5% atropine daily	8 days	Progesterone, 10 mg. Progesterone, 7 mg.†	5 10	5 10	101 72	+ +++	0 7

\* In this case the so-called "Pretreatment" and treatment were simultaneous.

† In this case the atropine treated animals lost weight while the controls gained during the pretreatment period. Hence it was decided to give 1 mg. of progesterone per 10 grams of body weight to make a fair comparison possible.



TABLE 5—Concluded

GROUP	PRETREATMENT	DURATION OF PRE-TREATMENT	TREATMENT	NUMBER OF ANIMALS		AVERAGE BODY WEIGHT	AVERAGE DEPTH OF ANESTHESIA	NUMBER OF DEATHS
				Males	Females			
IX	Progesterone, 6 mg.	1 hr.	Progesterone, 6 mg. 1 × 0.25 ml. 2% acetylcholine		6	70	+++	0
					6	71	+++	2
	0.25 cc. 2% acetylcholine	15 min.	Progesterone, 6 mg.		6	70	++	0
X	Progesterone, 6 mg.	40 min.	Progesterone, 4 mg.		6	46	+++	0
			1 × 0.4 cc. 2% acetylcholine		6	47	+++	6
XI	Progesterone, 4 mg.	*	Progesterone, 4 mg.	5	5	51	++	0
	1 × 0.2 cc. 2% acetylcholine			5	5	52	++	0
	7 × 0.2 cc. 2% acetylcholine	3 hrs.	Progesterone, 4 mg.	5	5	50	++	0
	Progesterone, 4 mg.	30 min.	1 × 0.2 cc. 2% acetylcholine	5	5	52	++	0
XII	Cervical vagotomy	3 hrs.	Progesterone, 7 mg.	5	5	70	+++	2
			Progesterone, 7 mg.	5	5	71	+++	8
XIII	Cervical vagotomy Cervical vagotomy Subdiaphragmatic vagotomy	*	Progesterone, 4 mg.	12	12	69	0	0
			Progesterone, 4 mg.	12	12	70	++	0
		3 hrs.	Progesterone, 4 mg.	12	12	68	+++	0
		3 hrs.	Progesterone, 4 mg.	12	12	70	+	0

against this drug, it appeared possible that their increased sensitivity to the anesthetic effect of progesterone is merely due to a loss of general resistance consequent to the utilization of their "adaptation energy" for inurement against atropine. Hence it appeared of importance to establish whether adaptation to other agents would likewise result in a sensitization to the anesthetic action of progesterone. In order to clarify this point, normal controls and rats adapted to formaldehyde and forced muscular exercise have been compared with regard to their progesterone resistance. Formaldehyde was administered subcutaneously in an aqueous solution to one group of animals while another group was forced to run daily in drum cages revolving at the rate of 18 revolutions per minute. Since during adaptation to strenuous treatment the animals do not gain in body weight as rapidly as untreated controls, the average body weight in the experimental groups was somewhat below



normal; consequently 1 mg. of progesterone was injected for each 10 grams of body weight rather than a fixed standard dose. The details of these experiments are summarized in table 6.

It is evident that while the animals acquired adaptation to formaldehyde and exercise they became more sensitive to the anesthetic action of progesterone. In the light of these observations it is quite possible that the rise in

TABLE 6

*Effect of adaptation to formaldehyde and forced muscular exercise on steroid hormone anesthesia*

PRETREATMENT	DURATION OF PRE-TREATMENT	TREATMENT	NUMBER OF ANIMALS		AVERAGE BODY WEIGHT	AVERAGE DEPTH OF ANESTHESIA	NUMBER OF DEATHS
			Males	Females			
	<i>days</i>				<i>grams</i>		
		Progesterone, 13 mg.	6	6	131	+	0
0.2 cc. 4% formaldehyde daily	15	Progesterone, 12 mg.	6	6	120	++	0
3 X 45 minutes exercise daily	15	Progesterone, 10 mg.	6	6	105	+++	2

TABLE 7

*Synergism between progesterone and volatile anesthetics*

PRETREATMENT	INTERVAL BETWEEN PRETREATMENT AND TREATMENT	TREATMENT	NUMBER OF ANIMALS		AVERAGE BODY WEIGHT	AVERAGE DURATION AND DEPTH OF ANESTHESIA
			Males	Females		
	<i>hour</i>				<i>grams</i>	
Progesterone, 3 mg.			6	6	39	Trace of anesthesia during 30 minutes
Progesterone, 3 mg.	1	Ether	6	6	40	+++ , anesthesia during 1 hour
		Ether	6	6	40	+++ , anesthesia during 5 minutes
Progesterone, 3 mg.	1	Chloroform	6	6	41	+++ , anesthesia during 1 hour
		Chloroform	6	6	39	+++ , anesthesia during 5 minutes

progesterone sensitivity seen in the rats chronically treated with atropine—which was described in the previous section of this paper—was not due to any specific action of this drug on the parasympathetic nervous system, but merely to the fact that during adaptation to atropine, all available “adaptation energy” became exhausted and consequently resistance to other agents (in this case progesterone) was decreased.



## SYNERGISM BETWEEN PROGESTERONE AND VOLATILE ANESTHETICS

In the case of combined administration of ether or chloroform with threshold doses of progesterone, the action of the volatile anesthetics is greatly increased and prolonged. This was observed in a series of experiments which were conducted in such a manner that not pretreated controls and rats given threshold doses of progesterone were simultaneously placed under a large dessicator in which they were exposed either to chloroform or to ether until they became deeply anesthetized. Table 7 summarizes the results of these experiments.

It is evident that doses of progesterone which in themselves elicited only a trace of an anesthetic effect changed the responsiveness to volatile anesthetics in such a manner that instead of their usual transitory action, they caused a prolonged deep anesthesia.

## SUMMARY AND CONCLUSIONS

Animal experiments with steroids which have hitherto not been examined in this respect confirm previous observations according to which only hormonally active steroid compounds produce anesthesia. Thus the diacetate of Reichstein's compound "J", which is hormonally inactive, has no anesthetic effect. Kendall's compound "A" which is an active cortical hormone, exhibits marked anesthetic potency. Vitamin activity does not confer an anesthetic action upon a compound since even large doses of ascorbic acid, *dl*- $\alpha$ -tocopherol, and calciferol proved completely devoid of this effect.

The observation that  $\Delta^5$ -acetoxy-pregnenolone has an anesthetic action appeared at first to represent an exception to the above mentioned rule since it was not known to have any hormonal effect. However, later experiments proved this substance to have a definite life-maintaining potency in the adrenalectomized rat. Since this compound is not known to occur in nature, it may well be the first instance of an "artificial cortical hormone" in the sense in which stilbestrol is an "artificial estrogen." Thus up to date among the many steroids examined, we know of no exception to what has been called "the fundamental law of steroid hormone anesthesia", since all hormonally active steroids have varying degrees of an anesthetic effect and conversely all steroid anesthetics possess some hormonal activity. The only apparent exception is 17-ethinyl testosterone, a hormonally active compound with which we have been unable to produce anesthesia up to the present time. However the compound is so insoluble that its inactivity as an anesthetic must most probably be regarded as due to the fact that sufficient quantities cannot be rapidly administered in solution.

Combined simultaneous treatment with two anesthetic steroids leads to a summation but not to a potentiation of their actions as shown by experiments in which D.C.A. and progesterone were given at the same time. On the other hand, there appears to be a true potentiation of the action of volatile anes-



thetics such as ether or chloroform when administered simultaneously with a steroid anesthetic, for instance progesterone.

Sensitivity to the anesthetic action of progesterone and D.C.A. is greater in young than in old rats.

Adrenalectomy sensitizes the organism to the anesthetic action of D.C.A., estradiol, testosterone and progesterone although it does not influence the anesthesia caused by chloroform, ether, or magnesium chloride. It is especially interesting to note that in these experiments D.C.A.—which is an adrenal cortical hormone capable of preventing adrenal insufficiency—proves more toxic in adrenalectomized than in the normal rat. This finding is discussed in connection with the clinical observations showing the simultaneous development of insufficiency and overdosage symptoms attributable to malfunction of the same endocrine gland.

Hypophysectomy sensitizes the organism to the anesthetic action of D.C.A. and progesterone just as adrenalectomy does. Since hypophysectomy causes selective atrophy of the adrenal cortex without significantly affecting the medulla, it is assumed that deficiency in adrenal cortical cells is responsible for the increased sensitivity seen after adrenalectomy and hypophysectomy.

Thyroidectomy does not influence the course of the anesthesia caused by progesterone or D.C.A. administration. This makes it highly improbable that the decrease in B.M.R. plays a significant rôle in the increased hormone sensitivity of the hypophysectomized rat.

Bilateral nephrectomy does not change the course of anesthesia caused by progesterone or D.C.A. Hence it is unlikely that renal excretion of the steroid hormones is of importance in the mechanism by which the body combats overdosage with such compounds.

Acetylcholine exerts no significant effect but short pretreatment with atropine increases and chronic pretreatment with this drug decreases resistance to the anesthetic action of progesterone. No definite conclusion can be drawn from these observations concerning the possible rôle of the vagus in the phenomenon of steroid hormone anesthesia. The decrease in resistance following prolonged daily treatment with toxic doses of atropine may well be due merely to the well known fact that while the organism acquires adaptation to a certain stimulus its resistance to other agents decreases. This interpretation is also supported by experiments showing that rats, which acquire a great deal of specific adaptation to toxic doses of formaldehyde or forced muscular exercise, also become particularly sensitive to progesterone anesthesia.

This investigation was supported by a grant from the Committee on Research in Endocrinology of the United States National Research Council. The author is greatly indebted to Professor T. Reichstein of the University of Basel, Switzerland for the "J"-diacetate, Professor E. C. Kendall of the Mayo Clinic for the compound "A", and to Professor Lewis F. Fieser of Harvard



University for purifying the  $\Delta^6$ -acetoxyprogesterone which was donated by Drs. Gregory Stragnell and Erwin Schwenk of the Schering Corporation of Bloomfield, New Jersey. Thanks are also due to Dr. A. Odell of Charles E. Frosst & Co. for the calciferol and to Mr. P. Blanc of Hoffman-LaRoche Ltd. for the ascorbic acid, the acetylcholine and the *dl*- $\alpha$ -tocopherol used in these experiments.

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# THE EFFECT OF SODIUM AMYTAL ON THE EMPTYING TIME OF THE NORMAL HUMAN STOMACH

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Received for publication June 16, 1941

In 1929 Olmsted and Giragossintz (1), while studying the effect of amytal anesthesia on glucose tolerance in dogs, suggested that amytal caused a prolonged contraction of the pyloric sphincter. They reasoned that this prevented the passage of glucose from the stomach into the intestine and so delayed glucose absorption. Dreyer and Hebb (2), working with decerebrated cats, found that the intravenous injection of sodium salts of the barbiturates continued to produce stimulation of gastric and intestinal movements until the doses became so large that respiration and circulation failed. Other workers, however, using different methods, did not confirm these findings. Quigley *et al.* (3), working with hypnotic doses in unanesthetized dogs, reported that gastrointestinal depression was produced by amytal and that it developed in the colon first and stomach last; recovery was in the opposite order. Gruber and his associates (4), (5) reported that sodium amytal decreased the general tonus and amplitude of the intestine in the intact unanesthetized dog and that it also depressed excised smooth muscle. Recently Gruber and Gruber (6) have reported further studies concerning the action of the barbiturates on the pyloric sphincter and the gastric musculature. These authors used dogs with permanent fistulae of the stomach and duodenum; one balloon was placed in the stomach and another in the pylorus. The barbiturates produced a diminution in the activity of the pyloric sphincter; the contractions were either inhibited completely or were decreased in force. It was further noted that the general tonus and muscular activity of the stomach was decreased in all the experiments. The time necessary for recovery was given from 38 to 198 minutes.

While considerable data have been presented relative to the effect of the barbiturates on various portions of the gastrointestinal tract, as far as the authors are aware no studies have been made of effect of therapeutic doses of sodium amytal on the gastric emptying time of the normal human stomach. It was deemed worth while to make such a study.

## METHODS

The subjects used in this investigation were young, healthy, adult males. Essentially the same method was used as reported in previous papers (7), (8). The meal



consisted of 15 grams of Quaker Farina which had been cooked down from a volume of 350 cc. to 200 cc. Fifty grams of barium sulfate were added so that the position of the meal could be determined fluoroscopically.

The subjects ate the test meal at about 8:00 a.m. No food had been eaten since the previous evening. They were instructed to relax mentally and physically as much as possible, although they were allowed to walk about the laboratory and corridors of the building. The time it took the meal to leave the stomach was determined to the nearest 15 minutes.

After the normal gastric emptying time had been established, the subjects were given a hypnotic dose (0.2 gram) of sodium amytal ten minutes before the meal was eaten. A punctured capsule containing the drug was taken with 100 cc. of water. It had been established previously that this amount of water did not influence gastric emptying with this type of meal.

In all instances several determinations were made and the average figure was used; the observations were made at exactly weekly intervals.

TABLE 1

*Effect of sodium amytal on the gastric emptying time*

SUBJECT	AVERAGE EMPTYING TIME (HOURS)				PER CENT CHANGE
	Control	Number of trials	After sodium amytal	Number of trials	
1	2.56	4	2.06	4	19.5 -
2	1.75	3	1.25	6	28.6 -
3	1.88	4	1.33	3	29.3 -
4	1.92	3	1.50	5	21.9 -
5	2.14	7	1.81	4	15.4 -
6	1.67	6	1.25	5	25.1 -
7	2.30	5	1.65	5	28.3 -
8	1.80	5	1.95	5	8.3 +
9	1.88	6	1.69	4	10.1 -
10	1.79	6	1.31	4	26.8 -
Average ....	1.97		1.62		19.7 $\pm$ 3.7*

\* Standard error.

### RESULTS

Table 1 gives the results obtained. The data clearly show that sodium amytal caused the stomach to empty faster. There was an average decrease in gastric emptying time of 19.7 per cent. This fact was statistically significant since the difference was more than 5 times the standard error.

### DISCUSSION

Since several competent observers have reported that the barbiturates cause a general relaxation of the tonus of gastric musculature and a decreased motor activity, it would not be expected that these compounds would cause the stomach to empty faster. It is, however, generally accepted that, while large doses of the barbiturates may cause a depression of smooth musculature, ordinary clinical doses produce no significant effect.



Presumably, most of the authors, who have studied the action of the barbiturates on the gastroenteric tract have used relatively large doses. In the work reported in this paper small doses were used, but while this might account for the fact that there was no depression of the smooth musculature of the stomach, it does not explain why the stomach emptied faster. As far as the authors are aware, no adequate explanation can be given at present for this finding. Several theoretical reasons might be given why gastric emptying was speeded up, but adequate proof could not be submitted for any of them. It is known that amytal may produce an imbalance of the autonomic nervous system, resulting in a depression of the cardiac vagus. Presumably this effect is not sufficiently marked with therapeutic doses to cause a similar action on the vagal fibers supplying the stomach. It is unlikely that therapeutic doses of sodium amytal have any important action on the pylorus, such as that reported several years ago by Olmsted and Giragossintz with large doses in dogs. As far as we are aware, however, no work has been reported on the effects of therapeutic doses of the barbiturates on the pyloric sphincter either on man or animals. This work should be done. The Grubers (6), in their recent work on the pyloric sphincter of dogs, used large doses. A possibility, although a rather remote one, is that the sedation produced by the sodium amytal reduced the effect of external stimuli; some of these might have caused inhibition of gastric motility and their removal might have allowed the stomach to empty faster. In a number of instances the subjects fell asleep and had to be awakened so that the necessary fluoroscopic examination could be made. According to Weitz and Vollers (9), however, sleep has but little if any effect on gastric motility in man.

In order to ascertain whether the alkali in the sodium amytal might be responsible in some measure for the decrease in gastric emptying time, seven subjects were given a hypnotic dose of amytal (0.2 gram). The same procedure was followed as when the sodium amytal was administered. No significant difference was noted between these results and those obtained with sodium amytal.

Since most of the reports in the literature emphasize the depressing effect of the barbiturates on the gastrointestinal tract, the authors feel that the results reported in this paper are of interest. They demonstrate the value of the use of therapeutic doses of drugs wherever possible in investigating the pharmacodynamic action of drugs and, if possible, using man as a subject.

#### SUMMARY AND CONCLUSIONS

Ten normal young adult males were given a hypnotic dose (0.2 gram) of sodium amytal. The gastric emptying time was decreased an average of 19.7 per cent; this was statistically significant. Nine of the ten subjects showed a decrease in gastric emptying; one showed an insignificant delay. Sodium amytal in therapeutic doses therefore hastens gastric emptying in the human being.



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# THE EFFECT OF VARIOUS AGENTS ON BLOOD COAGULATION TIME IN DOGS

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Received for publication June 25, 1941

A considerable number of substances have been reported to have the effect of lowering clotting time when systemically administered (1). Such an effect, if consistently obtained by a relatively non-toxic substance, would be of great clinical usefulness. The evidence supporting the effectiveness of these various agents has been obtained under a wide variety of conditions and the present report is a study of several such substances under one uniform set of conditions. The method consisted in the simple determination of clotting time of venepuncture samples in narcotized dogs. Such conditions can be made reasonably uniform and the results might be considered as having a suggestive bearing on the clinical usefulness of the compounds tested. The results, which were uniformly negative, have significance only for the specified conditions, however, and do not directly contradict results with other laboratory methods or any reliable demonstrations of clinical usefulness.

## PROCEDURE

Dogs were anesthetized with sodium pentobarbital (35 mgm. per kilogram intraperitoneally) and blood samples were withdrawn by venepuncture from the leg veins and the jugular veins. One cubic centimeter of withdrawn blood was expelled in two equally divided portions into Wassermann tubes maintained in a thermostatically controlled water bath at 38°C. At 15 to 30 second intervals the test tubes were rotated gently to determine if a clot had formed. When it appeared that coagulation had taken place, the test tubes were inverted and tapped on the bottom. When the blood no longer flowed down the side of the tube, clot formation was judged to have taken place. The coagulation time was taken as the interval between the first appearance of blood in the syringe and the formation of the clot and this was measured by a stopwatch. Results of the two determinations were averaged. All glassware was cleaned with chromic acid and freshly rinsed with saline just before use.

This procedure for determination of coagulation time is essentially that described by Dochez (2) and by Lee and White (3). Its reliability is primarily determined by uniformity in the manipulations of blood withdrawal, ejection and clot determination. After a number of preliminary trials this technique was considered to be satisfactory.

Each of the 4 leg veins and the 2 jugular veins were used in rotation in order to avoid as far as possible the effects which come from re-puncture of a vein. The clotting time on re-puncture is almost invariably lower but this variation can be minimized by pro-



longing the interval between punctures and by limiting the repeated punctures to the largest veins. Most of the experiments were performed on dogs used for the first time and occasionally when dogs were re-used an interval of 5 days or more was allowed between the experiments. Forty-two dogs were used in the course of the studies.

Moderate anesthesia was produced in all animals as it was believed desirable to eliminate any variations due to struggling and excitement. The experiments making up the control series with pentobarbital alone were carried out concomitantly with the experiments using drugs or were carried out on intervening days. Usually 3 or more determinations were made before the injection of the drug to be tested. After injection of the drug determinations were made every 15 or 30 minutes for about 3 hours.

This type of procedure is capable of yielding much more uniform results than any method which involves contact of the blood with the open tissue of a fresh stab wound. The number of variables introduced by such contact are necessarily much greater. In these studies some preliminary trials were made with the Bitti-Brooks technique using fresh stab wound incisions. The method was considered definitely less satisfactory. The Bitti-Brooks technique if applied to venepuncture samples would no doubt have been more satisfactory but did not seem to offer any advantages over the method which was adopted.

#### *Drugs tested*

Recent reports have indicated the effectiveness of *oxalic acid* as a systemic blood coagulant (4-9) although there has been one other recent observation which contradicted these findings (10). The favorable reports were based chiefly on experiments with rabbits and on clinical observations. The doses used in our studies were 1, 4 and 8 mgm. per kilogram intravenously (10 experiments), which were understood to cover the range originally suggested. The purity of the oxalic acid was checked by melting point and titration.

*Sodium citrate* has been reported to lower clotting time after systemic administration and it has been suggested that the effect is due to the destruction of blood platelets. In our studies the dosages used were 0.2 to 0.5 gram per kilogram intramuscularly in 50 cc. of water (5 experiments). This corresponds to the doses used in one of the most recent reports (11). Some of the earlier clinical reports involved the use of intravenously administered citrates but this route was not used in the present studies.

Adrenaline has been reported to cause a prompt, transitory decrease in blood coagulation time (12). In our studies the drug was injected in doses of 0.001 mgm. per kilogram intravenously (7 experiments) which corresponded to the doses of the original reports. Larger doses have been described as producing an increase in coagulation time.

Various commercial preparations have been used for the control of hemorrhage and two of the more prominent of these (an extract of blood platelets (13) and a hemostatic serum (14)) were included in these studies. The dosages used, respectively, were 6 mgm. per kilogram (7 experiments) and 0.2 cc. per kilogram (7 experiments). In both cases ampuled products were used and injected intravenously.

There have been suggestions that inhaled carbon dioxide markedly decreases blood coagulation time. There has also been a sporadically manifested impression that cyclopropane increases bleeding time during operations (15). Both of these gases, combined with oxygen, were administered in a rebreathing system for 30 minutes to dogs lightly narcotized with sodium pentobarbital. The mixtures, obtained with a Foregger metric gas anesthesia machine, were 15 per cent carbon dioxide—85 per cent oxygen and 20 per cent cyclopropane—80 per cent oxygen (2 experiments).

In an effort to evaluate the sensitiveness or responsiveness of the method, two substances with well-described anti-coagulant effects were included. These substances, heparin and Chlorazol-Fast Pink, were administered intravenously at 3 levels of dosage



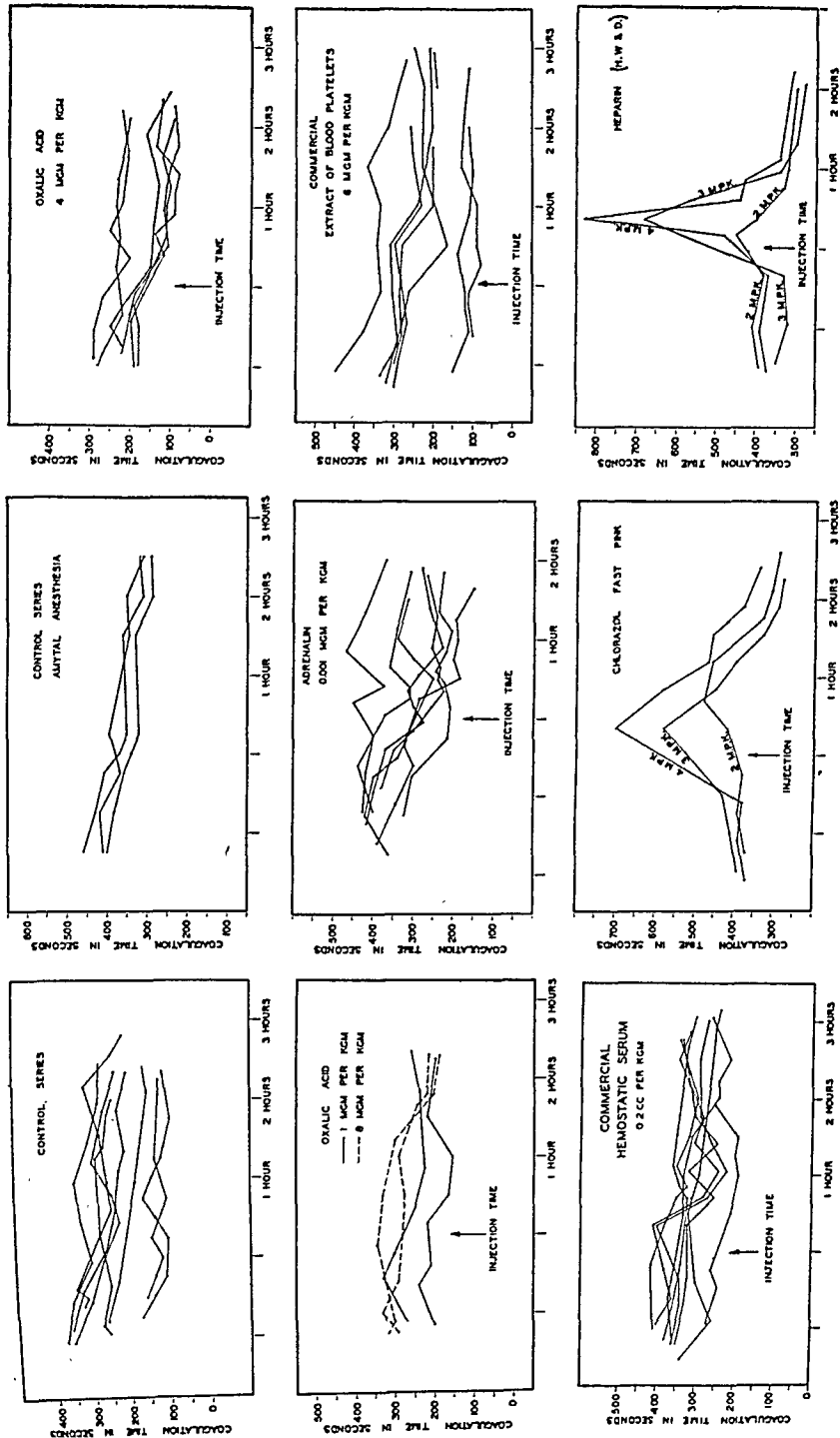


FIG. 1. EFFECT OF DRUGS ON BLOOD COAGULATION TIME



in order to determine the degree to which an increased response could be correlated with increased dosage (6 experiments). The heparin was the stock product supplied by Hynson, Westcott and Dunning. Some subsequent experiments with the specially purified heparin from the Connaught laboratories have given essentially the same type of results. The Chlorazol-Fast Pink was a product purified for physiological purposes by the Boots Drug Company, Nottingham, England.

### RESULTS AND DISCUSSION

Some of the results obtained are illustrated in figure 1 which shows the relation of coagulation time (in seconds) and experiment time (in hours). These graphs illustrate the extent of the variations obtained by this method.

In the control series, using sodium pentobarbital anesthesia alone, there was an average decrease of coagulation time amounting to approximately 20 per cent during the course of a 5 hour experiment. This moderate but definite decrease in coagulation time is most probably due to a very small amount of "tissue injury substance" produced by the repeated venepuncture. In the series using amytal anesthesia alone, the same type of decrease was noted and, in this case, the effect could not be attributed to a lessening of the depth of anesthesia such as frequently took place with the pentobarbital anesthesia.

The results obtained with oxalic acid, sodium citrate and the commercial coagulants did not indicate any significant decrease in coagulation time. Similarly there was not believed to be any significant decrease produced by adrenaline although the fluctuations were much wider in this case and there was a suggestive decrease in coagulation time by the end of the experimental period. The results obtained with carbon dioxide and with cyclopropane, similarly, did not show significant changes in coagulation time.

With the recognized anti-coagulant drugs, heparin and Chlorazol-Fast Pink, distinct increases in coagulation time were obtained as the dosages were successively increased through stages from 2 to 4 mgm. per kilogram. There was good correspondence of dosage and effect.

### SUMMARY

Blood coagulation time was determined in dogs anesthetized with sodium pentobarbital. Several drugs, previously reported to have systemic clotting effects, were found to be without distinct influence on blood coagulation time under these conditions. The same experimental conditions proved to be uniformly responsive to small, stepwise graded doses of anti-coagulant drugs.

The expense of this investigation was defrayed in part by a Grant from the Committee on Therapeutic Research of the Council on Pharmacy and Chemistry of the American Medical Association.

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# THE ACTION OF LUPINE ALKALOIDS ON THE MOTILITY OF THE ISOLATED RABBIT UTERUS

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Received for publication June 30, 1941

The lupine alkaloids, although widely distributed in nature and rather well known chemically, are in need of pharmacological investigation, the only one that has been rather completely investigated being sparteine. A number of years ago Couch (1) compared two hitherto little-studied members, *d*-lupanine and lupinine with sparteine, reporting on their comparative toxicity. More recently he added another, trilupine, to his list (2). Renewed interest in the therapeutics of sparteine was recently given to this alkaloid by Kleine (3), who reported favorably on the use of sparteine as a stimulant in the beginning stages of labor. Since small quantities of authentic samples of the above four alkaloids were available to us we were able to carry out comparative studies on uterine motility, which are herein reported. The present study was undertaken to ascertain (a) whether these alkaloids act like sparteine on the uterus, and (b) if stimulant, whether or not the order of their stimulating activity on the motility of the isolated uterus parallels that of their toxicity as reported by Couch (1).

## MATERIAL

Table 1 shows the lupine alkaloids used, together with their source, melting point, and the symbols by which they will be hereinafter referred to.

Figure 1 shows the probable structural formulae for the alkaloidal bases of: LB( $C_{16}H_{11}ON$ ), SS( $C_{16}H_{12}N_2 \cdot H_2SO_4 \cdot 5H_2O$ ), LDH( $C_{15}H_{11}ON_2 \cdot 2HCl \cdot H_2O$ ), and TLB( $C_{11}H_{11}O_2N_2 \cdot 2H_2O$ ).

## METHODS

Rabbits weighing 1.5 to 2.5 kg. were used in this study. They were killed by cervical blows and bleeding and the segments of uterus were removed and suspended immediately, one in each of two baths, in magnesium-free USP Locke-Ringer's solution (L-R). The chambers were of 100 cc. volume and were kept between 39.5 and 40.0°C. A constant supply of oxygen was bubbled through the solution. The chambers were arranged with a stopcock through the bottom of the water bath so that they could be emptied and refilled quickly with warm solution. The drugs were dissolved shortly before use in stock concentrations such that the desired concentration could be obtained by adding 1 cc. or less of drug solution to 99 cc. of L-R already in the chamber.

Isotonic contractions were recorded on slowly moving kymographs by light Harvard



TABLE 1

*Lupine alkaloids employed, together with their source, melting point and symbols used in referring to them*

NAME	SYMBOL	SOURCE	MELTING POINT °C.
Sparteine sulfate (USP IX).....	SS	Merck	
Sparteine disulfate (Lab.).....	SDS	Lupinus barbiger	264.5-265.5
Trilupine base (Lab.).....	TLB	Lupinus barbiger	127
<i>d</i> -Lupanine dihydrochloride (Lab.).....	LDH	Lupinus laxis	162-163
Lupinine base (Lab.).....	LB	Lupinus palmeri	68-69

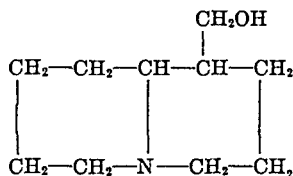
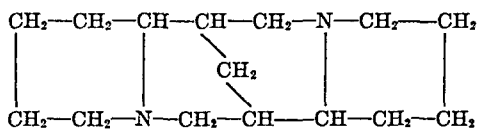
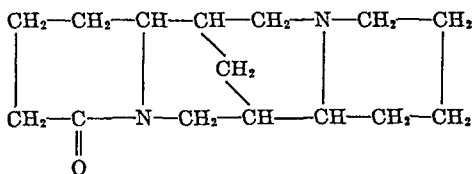
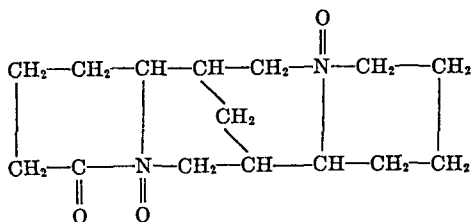
Lupinine<sup>1</sup>Sparteine<sup>1</sup>*d*-Lupanine<sup>2</sup>Trilupine<sup>3</sup>

FIG. 1. STRUCTURAL FORMULAE FOR THE LUPINE ALKALOIDS EMPLOYED

<sup>1</sup> Couch: Journ. Am. Chem. Soc., 58: 688, 1936.<sup>2</sup> Henry: The Plant Alkaloids, 145, 1939.<sup>3</sup> Couch: Journ. Am. Chem. Soc., 58: 1296, 1936.



heart levers. Each lever was accurately balanced and then loaded to give 0.5 gram to 4 gram stretch. The lever magnification was usually 2.5 times, occasionally 5 times.

Continuous records were taken, starting with L-R, adding a drug, continuing 10 to 15 minutes record, rinsing with L-R, continuing record 10 to 15 minutes, then adding drug, rinsing, etc. Drugs were usually added in ascending concentration, but several checks starting with the higher concentrations were performed. In most cases each of the 2 segments from each rabbit was subjected alternately to 2 drugs, for example: L-R; LR + SS 1:100,000; L-R; L-R + SDS 1:50,000; L-R; L-R + SS 1:20,000; etc. Simultaneously the other strip was subjected to L-R; L-R + SDS 1:100,000; L-R; L-R + SS 1:50,000; L-R; L-R + SDS 1:20,000; etc. By this method any difference in reactivity which might exist between the pair of segments was ruled out.

### OBSERVATIONS

Thirty-four uterine segments from 17 rabbits were used, and a total of 226 individual tests of activity of the lupine alkaloids was performed. Of the uteri 11 were of the virgin type, 4 rutting, and 2 pregnant. The 226 individual tests were distributed as follows: SS, 25; SDS, 110; TLB, 23; LDH, 18; LB, 50. The number of tests with SDS is highest since TLB, LDH, and LB were usually tested against it for comparison. The concentrations routinely employed were: 1:500,000; 1:100,000; 1:50,000; 1:20,000; 1:5,000; 1:1,000.

The uterine segments were stimulated by any of the lupine alkaloids in proper concentration. The typical responses have been divided arbitrarily into 5 categories to form a basis for comparison (fig. 2). Response *A* is the effect of minimal concentrations producing stimulation. *B* is the response to concentrations giving maximal stimulation; *C*, to concentrations producing maximal stimulation, but not maintaining this at its peak level; *D*, to concentrations inhibitory to motility, though with preliminary increase in tone and sometimes rate. The response *E* differs from *D* in that the preliminary increase in tone is greater and is maintained for a much longer time. This condition may be interpreted as spasticity instead of depression of motility, or it may be both. It is peculiar to higher concentrations of LB, and to higher concentrations of SDS on pregnant uteri.

Table 2 is a summary of experiments on lupine alkaloids showing the concentrations required to produce responses *A*, *B*, *C*, *D*, or *E*.

No two pairs of uterine segments reacted exactly alike. The pregnant segments typically showed a normal motility in which there were large contractions, usually with small superimposed ones, while the virgin showed small, often single, contractions. On stimulation of the pregnant type, the contractions were larger and usually more prolonged; stimulation of the virgin type produced larger contractions but seldom showed much tendency towards prolonged contraction except with higher concentrations of LB. The rutting type was intermediate in all respects.

Sharp lines of demarcation cannot be drawn between the five categories of responses, there being all intermediate conditions. The sensitivity of one pair of segments was often considerably lower than that of another pair to a



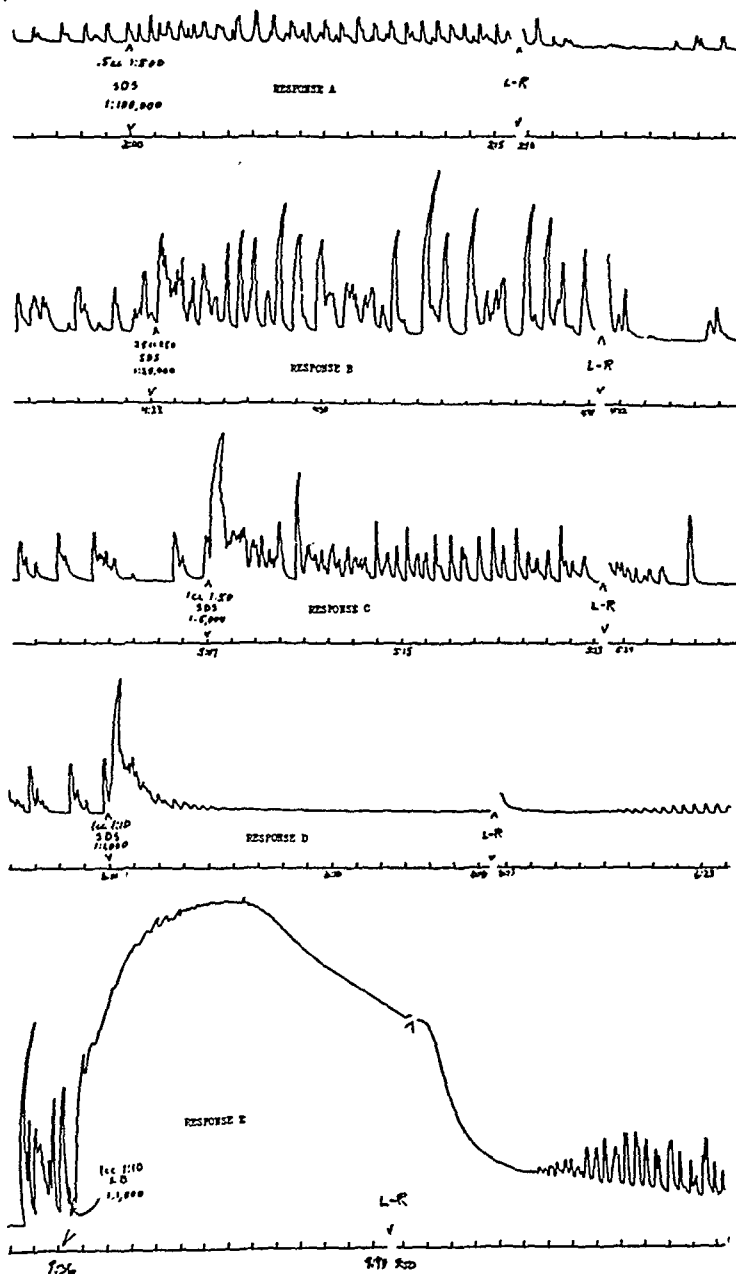


FIG. 2. TYPICAL UTERINE RESPONSES TO THE LUPINE ALKALOIDS



particular concentration of any one of the alkaloids, yet the ratio between the concentration required to produce maximal stimulation (response *B*) and that just sufficient to produce stimulation (response *A*) was rather constant.

TABLE 2

Summary of experiments, showing concentrations in each experiment at which responses *A*, *B*, *C*, *D*, and *E* occurred

DRUG	ANIMAL	TYPE OF UTERUS	CONCENTRATIONS REQUIRED TO PRODUCE RESPONSES:				
			<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	<i>E</i>
SS	VI	Virgin	1:100,000	1:20,000	1:5,000	1:1,000	
	XVIII	Virgin	1:100,000	1:50,000	1:20,000	1:5,000	
	XIX	Virgin	1:100,000	1:20,000	1:5,000	1:1,000	
SDS	I	Rutting	1:200,000	†	†	†	
	III	Virgin	1:100,000	†	†	1:1,000	
	VI	Virgin	1:100,000	1:20,000	1:5,000	1:1,000	
	VII	Virgin	1:50,000*	1:20,000	1:5,000		
	VIII	Virgin	1:50,000*	1:20,000	1:5,000	1:1,000	
	IX	Virgin	1:50,000*	1:20,000	1:5,000		
	XVI	Virgin	1:50,000*	1:20,000	1:5,000		
	XVIII	Virgin	1:100,000	1:50,000	1:20,000	1:5,000	
	XIX	Virgin	1:100,000	1:50,000	1:5,000	1:1,000	
	XX	Virgin	1:50,000*	1:20,000		1:1,000	
	XXI	Virgin	1:100,000	1:20,000	1:5,000	1:1,000	
	I	Rutting	1:200,000	†	†	†	
	X	Rutting	1:50,000*	1:20,000	1:1,000		
	XIII	Rutting	1:50,000*	1:20,000	1:5,000	1:1,000	
	IV	Pregnant	1:100,000	1:50,000	1:5,000		1:1,000
TLB	V	Pregnant	1:100,000	1:50,000	1:20,000		1:5,000
	III	Virgin	1:20,000	1:1,000			
	XX	Virgin	1:20,000	1:5,000			
	XXI	Virgin	1:5,000	1:1,000			
LDH	XVII	Rutting	1:5,000	1:1,000			
	XVI	Virgin	1:5,000	1:1,000			
	XVII	Rutting	1:5,000	1:1,000			
LB	IV	Pregnant	1:10,000	1:1,000			
	VII	Virgin	1:20,000	1:20,000	1:5,000		†
	VIII	Virgin	1:20,000	1:5,000			1:1,000
	IX	Virgin	1:20,000	1:5,000	1:1,000†		
	X	Rutting	1:20,000	1:5,000			1:1,000
	XIII	Rutting	1:20,000	1:5,000			1:1,000
	V	Pregnant	1:50,000	1:20,000	1:5,000†		1:1,000

\* 1:100,000 not used.

† Maintained high tone.

‡ Concentration which might have produced this effect not used.

*Sparteine sulfate*. It will be seen from an examination of Table 2 that there was little or no difference between the concentration of SS and SDS required to produce any given effect, except that the response *E* was not ob-



tained with SS. This, however, would not be expected since SS was not used in the higher concentrations on pregnant segments.

*Sparteine disulfate.* While the lowest concentration producing stimulation (response A) was from 1:200,000 to 1:50,000, the most reliable concentration is 1:100,000; in those cases where 1:50,000 is listed as minimal, no intermediate concentration between 1:50,000 and 1:500,000 was used. Response A usually showed slight stimulation of amplitude and rate; often there was stimulation only of amplitude or of rate. Maximal stimulation (response B) was obtained with concentrations of 1:50,000 on some segments; others required 1:20,000. Stimulation here was evidenced particularly as increase in amplitude, often 3 to 4 times as great as normal, with or without increase in rate. Such stimulation was maintained for the usual 15 minute period of observation. Response C was produced in some segments by a concentration of 1:20,000; others required 1:5,000. In those cases in which increased tone was evident, this decreased after several minutes toward the original level. Response D was obtained in some virgin type segments with a concentration of 1:5,000; others required 1:1,000. The amplitude of contractions rapidly decreased, frequently with complete cessation of motility within 5 minutes, and at the end of 15 minutes the tone was usually no greater than before administration of the drug. Response E was produced in the pregnant type by the same concentrations. This differed chiefly from response D in that the increased tone had dropped off only slightly by the end of 15 minutes. Return to original level of activity was obtained in L-R after any concentration of this drug employed, although it was much slower after the higher concentrations.

*Trilupine base and d-lupanine dihydrochloride.* Response A was produced by concentrations of 1:20,000 to 1:5,000; response B by 1:1,000. Concentrations above 1:1,000 were not employed. The responses A and B were essentially similar to those produced by SDS in about  $\frac{1}{5}$  these concentrations. Return to original level of activity was easily obtained in L-R.

*Lupinine base.* A minimal concentration of 1:50,000 was required to produce response A in one (pregnant) segment and 1:20,000 in others. Response B was produced in some segments by 1:20,000 and in the others by 1:5,000. Responses A and B were essentially similar to those produced by SDS in about  $\frac{1}{5}$  these concentrations. Response C was produced by 1:5,000 or 1:1,000 in a few cases, but in most of these the tonus remained rather high. Response E was produced in most cases by a concentration of 1:1,000. Here the tonus increase was at least as great as that produced by SDS on pregnant uteri, regardless of whether the LB was used on pregnant or non-pregnant uteri. It had a greater tendency than had SDS for producing spasticity with the higher concentrations. Return to original level of activity could be obtained in L-R, though often difficult and sometimes only partial after LB 1:1,000.



## DISCUSSION

On comparing the data in tables 3 and 4 it will be seen that, (1) TLB is only  $\frac{1}{16}$  as active as SDS as a stimulant of uterine motility, while it is about  $\frac{1}{2}$  as toxic as sparteine sulfate; (2) LB is about  $\frac{1}{2}$  as active as SDS as a stimulant of uterine motility, and is slightly more toxic than sparteine sulfate; and (3) LDH is only  $\frac{1}{16}$  as active as SDS as a stimulant of uterine motility, and is slightly more toxic than sparteine sulfate. These comparisons indicate that while all four drugs are capable of stimulating uterine motility SDS would probably be the safest clinically as an abortifacient, TLB not over  $\frac{1}{2}$  as safe as SDS, LB not over

TABLE 3

*Average concentrations of lupine alkaloids producing responses A and B*

DRUG	A	B
	mg./kg.	mg./kg.
SS	9	30
SDS	9.5	41
LB	45	150
TLB	125	800
LDH	167	1,000

TABLE 4

*Data from Couch (1, 2) comparing toxicity of lupine alkaloids injected intraperitoneally in guinea pigs*

DRUG	TOXIC	M.L.D.
	mg./kg.	mg./kg.
Lupanine hydrochloride. . . . .	24-28	25-29
Lupinine base. . . . .	25-28	28-30
Sparteine sulfate. . . . .	27-55	42-55
Trilupine base. . . . .	175-210	200-225

$\frac{1}{2}$  as safe, and LDH not over  $\frac{1}{16}$  as safe. Indeed, since Kleine (3) used approximately 3 mg. of sparteine sulfate per kilogram of body weight (assuming a body weight of 70 kg.) it is doubtful that stimulation of uterine motility could occur if sub-lethal doses of LDH were employed clinically.

## SUMMARY

1. A study of sparteine sulfate USP IX, and samples of sparteine disulfate, *d*-lupanine dihydrochloride, trilupine and lupinine base on the motility of isolated rabbit uterus showed that with effective concentrations there was stimulation of amplitude, rate, or tone, or a combination of these, followed in some higher concentrations by depression of amplitude or rate, with or without decrease in tone towards original level.



2. Return to previous level of activity could be obtained in Locke-Ringer's solution after any of these drugs in the concentrations used (up to 1:1,000).

3. The qualitative effects of these alkaloids upon motility of the isolated rabbit uterus were quite similar in the virgin, rutting and pregnant types, but the magnitude of response was greatest in the pregnant and least in the virgin type. The pregnant, and less so the rutting type, showed a stronger tendency toward increases in tonus with the higher concentrations.

4. Little or no difference was found between the activity of sparteine sulfate USP IX and sparteine disulfate, laboratory sample.

5. Sparteine disulfate 1:100,000 was the minimal concentration producing stimulation; 1:50,000 or 1:20,000 produced maximal, maintained stimulation; 1:20,000 or 1:5,000, stimulation not maintained at maximal level more than several minutes; and 1:5,000 or 1:1,000 produced stimulation followed almost immediately by depression of amplitude even to cessation of motility.

6. Trilupine base and *d*-lupanine dihydrochloride produced effects qualitatively similar to those produced by sparteine disulfate, but were only about  $\frac{1}{15}$  as effective. No depressant effects were observed. Lupinine base was only about  $\frac{1}{5}$  as effective a stimulant as sparteine disulfate; however, the increase in tone was greater, and in high concentrations (1:5,000 or 1:1,000) it tended to produce prolonged spasticity.

7. Comparison of the results of this study with studies on comparative toxicities, indicate that, if used clinically as ecboics, sparteine sulfate would probably be the safest, trilupine base about  $\frac{1}{3}$ , lupinine base about  $\frac{1}{5}$ , and *d*-lupanine dihydrochloride only about  $\frac{1}{15}$  as safe as sparteine disulfate.

The author wishes to thank Dr. James Fitton Couch (formerly of the Pathological Division, Bureau of Animal Industry, Department of Agriculture, Washington, D. C., and now at its field station in Philadelphia, Pennsylvania) for his generosity in supplying the lupine alkaloids used in this study.

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# EFFECT OF ATABRINE UPON EXPERIMENTAL CYSTICERCOSIS OF MICE

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Received for publication July 7, 1941

Although cysticercosis has long been known in both man and animals, no specific treatment for the condition has yet been described. Surgical removal of the cysts, when these are lodged in accessible sites, is the favored procedure in therapy, but when their removal by operation is not possible, little or nothing beyond symptomatic treatment remains. Recently, one of us has described an antagonistic action of the acridine derivative, atabrine, upon *Hymenolepis fraterna* (1), a cestode which dwells in the villi and subsequently in the lumen of the small intestine of the albino mouse. Generally, by the administration of atabrine for from one week to ten days, mice can be freed of this infection. The adult worms are deeply stained by the drug after a day or so, and usually, when eliminated, reveal more or less degeneration. Even the larval worms in the villi eventually appear to be destroyed by the drug. The fact that atabrine is absorbed into the blood of treated animals and continues to experience the entero-hepatic cycle of the bile salts for a considerable period thereafter led the authors to try atabrine in experimental cysticercosis (*Cysticercus fasciolaris*) of the mouse (2). This infection is largely or entirely confined to the mouse liver, and the parasites are therefore well exposed to the absorbed drug. In the present paper the results of this trial of atabrine upon cysticercosis of the mouse are presented. As will be shown, atabrine usually retards the growth of the cysticerci, and frequently prevents entirely their development.<sup>1</sup>

## METHODS

*The infection and examination of mice.* Mice weighing 20 to 25 grams were infected with 500 oncospheres of *Taenia crassicolis*. The oncospheres were suspended in a volume of 0.1 cc of physiological salt solution and delivered by syringe through a blunted 22 gauge hypodermic needle lowered carefully into the retropharynx of each animal. The treated animals along with control mice were autopsied four weeks after infection and the livers examined for the presence of cysticerci.

*The administration of atabrine.* The atabrine, in water solution or suspension,

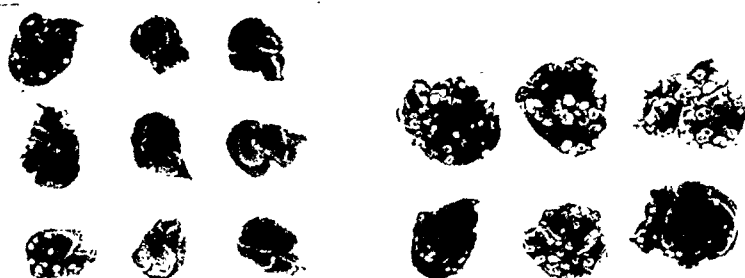
<sup>1</sup> The atabrine used in this work was supplied by the Winthrop Chemical Company of New York City.



was administered by syringe through a blunted hypodermic needle lowered into the mouth. The treatment was carried on for two days before the animals were infected, 5 mg. of the drug being given daily. Beginning on the day after infection, 5 mg. of the drug were given on alternate days for two weeks, and then, for the two succeeding weeks, twice this dose was given at the same interval.

TABLE 1  
*Number of cysticerci counted on livers of atabrine-treated mice and control mice*

	MOUSE NUMBER	CYSTS PRESENT			MOUSE NUMBER	CYSTS PRESENT		
		Living	Dead	Total		Living	Dead	Total
Atabrine-treated mice	1	0	0	0	12	2	0	2
	2	0	11	11	13	2	32	34
	3	0	17	17	14	7	43	50
	4	0	18	18	15	11	12	23
	5	0	19	19	16	20	0	20
	6	0	24	24	17	25	3	28
	7	0	33	33	18	25	20	45
	8	0	48	48	19	28	32	60
	9	0	48	48	20	30	0	30
	10	0	54	54	21	30	27	57
	11	0	79	79	22	101	3	104
Control mice	31	62	14	76	36	135	18	153
	32	66	23	89	37	137	0	137
	33	87	0	87	38	139	14	153
	34	117	0	117	39	153	0	153
	35	134	23	157	40	169	0	169



Atabrine treated

Control

FIG. 1. LIVERS OF ATABRINE-TREATED MICE AND OF CONTROL MICE FOUR WEEKS AFTER INFECTION WITH 500 ONCHOSPHERES OF *TAENIA CRASSICOLLIS*

#### RESULTS

Altogether, 90 mice were used in this work, of which 60 were treated with atabrine and 30 were kept as controls of the infection. The experiment was performed several times, with different lots of mice. The last trial involved



32 animals. These were infected as described above, and 22 were treated according to the previously outlined schedule. The results obtained with this group will be presented in detail.

At autopsy, four weeks after infection, all the control mice were found to be heavily infected, their livers studded and distended with many living cysticerci. Among the twenty-two atabrine-treated mice, in contrast, eleven revealed no living cysts whatsoever, and ten of the remaining eleven presented only a few living cysts. In the case of only one of the treated mice were the cysts as large and as numerous as they were in the control mice. On the livers of 16 of the treated animals dead cysts were observed, whereas on the livers of only 5 of the control mice were dead cysts present. The appearance of the livers of representative treated animals and of representative controls is shown in figure 1. In table 1 the actual number of living cysts and of dead cysts counted on the livers of all the mice are presented.

#### DISCUSSION

The experiment presented above shows rather clearly that atabrine either prevents or retards the development of *Cysticercus fasciolaris* in the albino mouse. It should be borne in mind, however, that the effect obtained may be largely prophylactic, since the atabrine was first given prior to the infection of the mice. In additional experiments which have not been included in this report, the administration of the drug was withheld for several weeks until the cysticerci had become established. Under this circumstance, the effect of the atabrine was distinctly less, and usually was not significant. The application of drug therapy to human cysticercosis or to a related cestode infection, echinococcus (hydatid) disease, must evidently await the discovery of a substance more effective than atabrine appears to be upon the established cyst. It seems possible that one of the other acridine derivatives might serve this purpose.

#### CONCLUSION

The development of *Cysticercus fasciolaris* in the mouse can be prevented or retarded by the administration of atabrine.

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# THE DEPRESSION OF EXPERIMENTAL POLYCYTHEMIAS BY VARIOUS SUBSTANCES IN DOGS, RABBITS, AND MAN

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Received for publication July 7, 1941

In 1935, Marshall (1) showed that cobalt polycythemia in rats could be partially reduced by daily injections of a liver extract but was unaffected by the feeding of whole liver. The failure of liver feeding to reduce cobalt polycythemia in this species has been confirmed by Anderson, Underwood, and Elvehjem (2). We have reported previously (3) that the oral administration of whole liver (raw) to dogs is effective in depressing polycythemias induced by cobalt feeding or daily exercise. Subsequently (4) we found that orally administered choline hydrochloride was as effective as liver in reducing experimental polycythemia in dogs, and we stated our belief that choline is the active constituent of liver here concerned. Later (5) it was found that atropine antagonized the hemopoietic depressant action of both choline and liver in polycythemic dogs, and that certain vaso-dilator drugs were also effective in reducing polycythemia. We explained the action of these substances by assuming that they dilate bone marrow arterioles, thereby improving the blood supply to this tissue and diminishing the local anoxia which is probably the immediate stimulus to polycythemia. Recently (6) we have added support to this theory by *producing* experimental polycythemia in dogs, rabbits, and man by the daily administration of ephedrine, a vasoconstrictor drug.

The present investigation was designed primarily to test the effectiveness of three different vasodilator drugs in reducing polycythemia in animals. The drugs chosen were aminophylline (theophylline ethylene diamine), mecholyl (acetyl  $\beta$ -methyl choline), and mannitol hexanitrate. In the second place, it was desired to see whether experimental polycythemia in *rabbits* could be reduced as readily as in dogs. We have found no reports in the literature concerning the depression of polycythemia in this species by liver or vasodilator drugs.

## METHODS

The animals used in this investigation consisted of 6 dogs and 9 rabbits which were fed an adequate constant diet and allowed water *ad libitum*. Two of the rabbits and one dog were splenectomized, to determine whether the spleen was a factor in the results observed in our experiments.



Red blood cell counts were made by the usual method and hemoglobin percentages were determined with a Hellige hemometer. Total leucocyte counts also were made frequently on dog's blood. Blood was drawn by syringe from the saphenous veins of the dogs, only at times when the animals were unexcited and at least 17 hours after the previous administration of drugs. We wish to emphasize that we were not studying the *immediate* effects of any of the drugs, but rather the *chronic* changes produced on the blood. Blood was drawn from rabbits directly into diluting pipettes from the site of puncture of a marginal ear vein.

Polycythemia was produced in 5 rabbits by the daily subcutaneous injection of 10 mg. of cobalt (40 mg.  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ). The increases in red cell count thus produced ranged from 18 to 51 per cent, depending somewhat upon the duration of cobalt administration.

Ephedrine sulfate was administered daily to produce a polycythemia in both dogs and rabbits according to methods recently reported by us (6, 7). Dogs were given ephedrine in daily doses of 2.5 to 5 mg. per kilogram by stomach tube. Rabbits received subcutaneous injections of 45 mg. of ephedrine sulfate daily. Maximal increases in red cell count in both dogs and rabbits were usually reached within 2 weeks by the daily administration of ephedrine. These increases ranged from 13 to 22 per cent above normal.

Benzedrine sulfate was also used to produce polycythemia (7). The daily dose for both dogs and rabbits was 10 mg., orally, and the increases of erythrocyte counts obtained in these experiments ranged from 15 to 17 per cent.

Three vasodilator drugs were chosen for trial of their effectiveness in reducing experimental polycythemia. Each drug was selected as a representative of a certain pharmacological group, viz.: a choline derivative, a xanthine derivative, and an organic nitrate. Mecholyl chloride was given to both polycythemic dogs and rabbits in a daily dose of 0.5 mg. per kilogram by subcutaneous injection. Aminophylline was given orally to either species in a daily dose of 100 mg. Mannitol hexanitrate was administered orally in a dose of 30 mg. daily to both dogs and rabbits.

In addition, we administered choline hydrochloride by mouth to 3 rabbits with cobalt polycythemia in a daily dose of 100 mg. to see whether this drug was effective by the oral route in this species.

Ephedrine polycythemia was produced in one human subject by the daily ingestion of 50 mg. of the sulfate (7). Slightly cooked liver was then eaten for 4 days (one-quarter pound daily) to see whether liver would reduce experimental polycythemia in a human subject, as it does in dogs (3).

## RESULTS

Acetyl  $\beta$ -methyl choline, 0.5 mg. per kilogram daily, was injected subcutaneously into 4 rabbits with cobalt polycythemia, one normal and 2 splenectomized rabbits and one dog having ephedrine-induced polycythemia, and 2 dogs with polycythemia induced by benzedrine (fig. 1). In spite of continued hemopoietic stimulating measures the injection of mecholyl into these animals resulted in a significant decrease of erythrocyte number in every case, within 3 or 4 days. Although total leucocyte counts are not shown in the graph, they were followed in the dogs, and did not change significantly during the reduction of red cell counts by mecholyl. Hemoglobin percentages, however, decreased with the red cells.

Aminophylline was given orally in daily doses of 100 mg. to 2 dogs having



ephedrine-induced polycythemia, to 2 dogs with "benzedrine" polycythemia, and to 2 splenectomized rabbits in which polycythemia was induced by ephedrine and benzedrine. Within 3 days, and in spite of continuation of ephedrine or benzedrine, the erythrocyte numbers of all of the six animals had

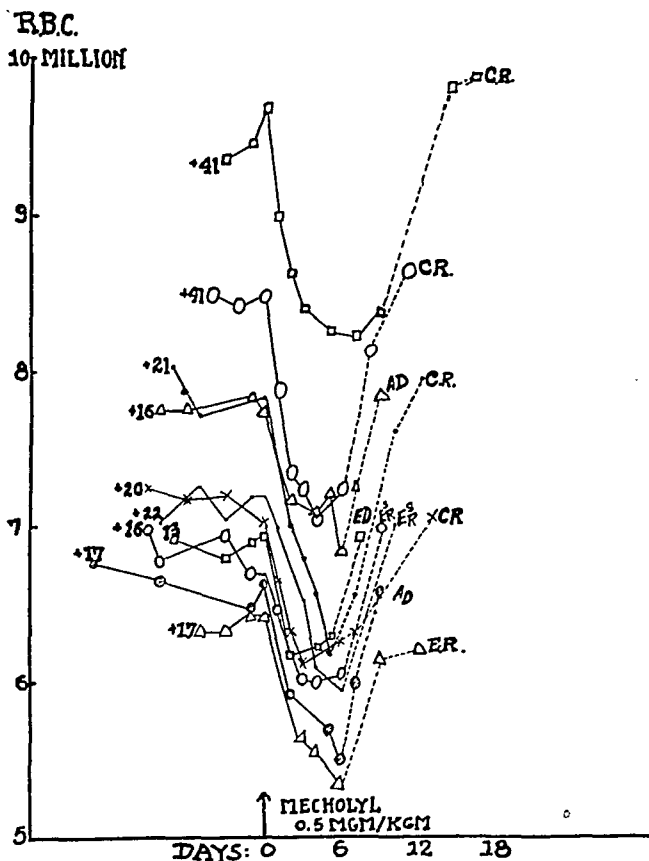


FIG. 1. EFFECT OF DAILY SUBCUTANEOUS INJECTION OF 0.5 Mgm. PER KILOGRAM ACETYL  $\beta$ -METHYL CHOLINE CHLORIDE ON 10 POLYCYTHEMIC ANIMALS

Dashed lines show return of polycythemia after cessation of injections. Figures at start of each line show the degree of polycythemia for each animal in terms of percentage increase of erythrocyte count above normal. C.R. = cobalt rabbit; E.R. = ephedrine rabbit; A.D. = amphetamine dog; E.D. = ephedrine dog; S = splenectomized.

been reduced considerably (fig. 2). The administration of the same dose of aminophylline had no effect on the red blood cell counts of normal dogs, similarly studied.

Aminophylline (100 mg. daily by stomach tube) had no effect on the red



cell numbers of 3 rabbits which had marked polycythemia developed by cobalt injection.

Mannitol hexanitrate was administered by stomach tube in a daily dose of 30 mg. to 5 rabbits which had cobalt polycythemia and one splenectomized dog with benzedrine polycythemia (fig. 3). Erythrocyte counts on all of these animals were found to be reduced within 3 or 4 days, despite continuation of the polycythemic stimulus. The same dose of mannitol hexanitrate had no effect upon the erythrocyte numbers of normal rabbits, similarly studied.

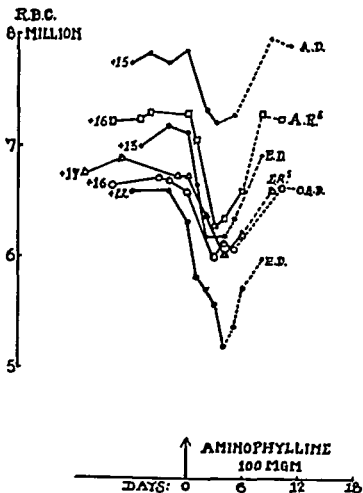


FIG. 2. EFFECT OF ORAL ADMINISTRATION OF AMINOPHYLLINE UPON THE RED CELL COUNTS OF 6 POLYCYTHEMIC ANIMALS

Dashed lines indicate discontinuation of drug. Figures at left of each line show degree of polycythemia as per cent increase of erythrocytes over normal.

Choline hydrochloride (100 mg. daily) was given by stomach tube to 3 rabbits which had cobalt polycythemia. This drug proved to be as effective as mecholyl in reducing the erythrocyte numbers of these animals. The experiments are not shown here because we have previously demonstrated the effectiveness of orally administered choline in depressing polycythemia in dogs (4, 5).

Slightly cooked calves' liver (one-quarter pound daily) was ingested by one



human subject who had experimental polycythemia induced by ephedrine (50 mg. daily). As will be seen in figure 4, the eating of liver caused a gradual reduction of his red cell count and hemoglobin percentage to approximately

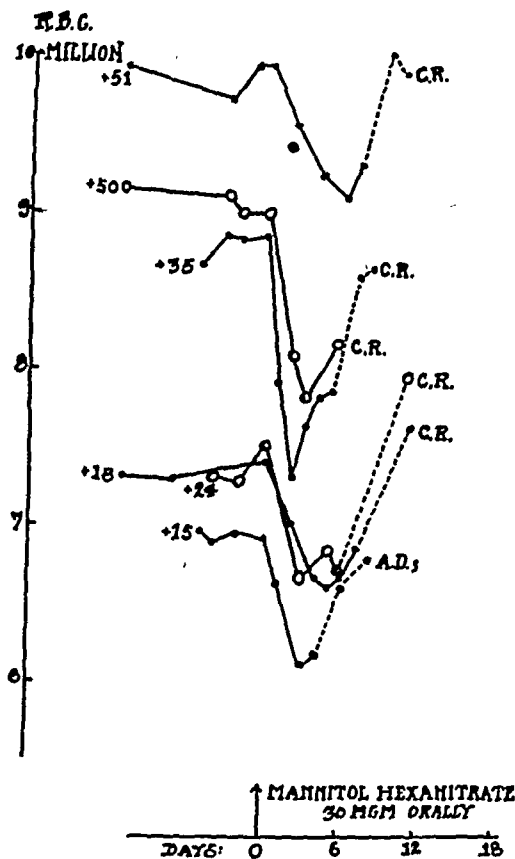


FIG. 3. DEPRESSION OF POLYCYTHEMIA BY MANNITOL HEXANITRATE

Figures at left show percentage increase of red cell count above normal, before depression by the drug. C.R. indicates rabbits which received cobalt throughout the experiment, and A.D. designates an amphetamine-treated dog which is splenectomized.

normal values which were reached after 4 daily doses of liver. At this time both liver and ephedrine were discontinued (dashed lines, figure 4), following which the erythrocyte number climbed toward a polycythemic value and slowly returned to normal within about 9 days. Total leucocyte counts remained fairly constant throughout the experiment.



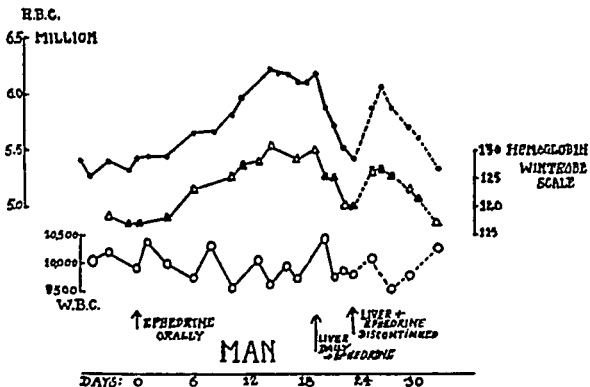


FIG. 4. DEPRESSION OF EPHEDRINE-INDUCED POLYCYTHEMIA IN MAN BY ONE-QUARTER POUND OF CALVES LIVER DAILY

Dashed line indicates discontinuation of both liver and ephedrine. Solid dots represent the erythrocyte counts; open dots, leucocyte counts; triangles, hemoglobin percentage (100 per cent = 14.5 grams per 100 cc. of blood).

#### DISCUSSION

Acetyl  $\beta$ -methyl choline appears to be effective in reducing all types of experimental polycythemia used in this work (fig. 1). In a previous investigation (5) we have shown this drug and the ethyl ether of  $\beta$ -methyl choline to be effective in depressing cobalt polycythemia in dogs. Similarly, mannitol hexanitrate (fig. 3) seems to be effective in depressing high red cell counts produced either by cobalt or by a vasoconstrictor drug (benzedrine). Aminophylline, while effective against polycythemia caused by vasoconstrictor drugs (fig. 2), has no significant effect on cobalt polycythemia in rabbits so far as we could see in experiments not shown here. The reason for this may be that aminophylline is a weaker vasodilator substance or that cobalt polycythemia as we have produced it in rabbits (we did not use cobalt-fed dogs), is harder to reduce by vasodilator substances. The latter suggestion would appear possible since none of the substances used in this investigation were successful in *completely* reducing the highest cobalt polycythemias shown in our figures, and since the mechanism of cobalt polycythemia is not definitely known, although the author has suggested previously (8) that cobalt may stimulate erythropoiesis indirectly by interfering with a cellular respiratory function of vitamin C.



The effectiveness of mannitol hexanitrate in depressing experimental polycythemia agrees with our previous positive results with sodium nitrite, in demonstrating that polycythemia may be reduced by drugs whose action is primarily one of direct vasodilation which is not antagonized by atropine.

Our finding (not shown) that orally ingested choline reduces cobalt polycythemia in rabbits is interesting in view of the reports (1, 2) that orally administered liver or liver extract is ineffective against this type of polycythemia in another rodent, the rat.

We noticed in this work that cobalt polycythemia in the rabbit required eight or more weeks to develop fully. The maximum increase of red cell counts obtained was about 50 per cent which is a higher value than any that we have found reported for this species in the literature.

The mechanism by which liver and vasodilator drugs depress excess hemopoiesis in polycythemic animals may be presumed to be the same for all such substances that are effective. They all appear to require about 3 or 4 days to produce a maximal decrease of red cell count in polycythemic animals. They *do not* decrease the red cell count in *normal* animals in the doses used. The one general property possessed by all of them is a vasodilating action. The author therefore believes that the probable mechanism of action, as stated previously (5), is through vasodilation and improvement of blood (and oxygen) supply to bone marrow. This reduces the local anoxia which is presumably the immediate stimulus to polycythemia.

Among the substances found to be more or less effective in depressing experimental polycythemia in this and previous (4, 5) work are: raw liver, choline hydrochloride, acetyl-choline, acetyl  $\beta$ -methyl choline, ethyl ether of  $\beta$ -methyl choline, sodium nitrite, mannitol hexanitrate, and theophylline ethylene diamine.

Choline hydrochloride has been tried without success in human *polycythemia vera* by Carpenter (9) and by Meyer and Thewlis (10). The drug was administered orally in doses of about 0.2 gram three times daily.

#### CONCLUSIONS

The daily subcutaneous injection of 0.5 mg. per kilogram of acetyl  $\beta$ -methyl choline significantly depressed the high red blood cell counts in 4 rabbits with cobalt polycythemia, 2 splenectomized and one normal rabbit with ephedrine-induced polycythemia, 2 dogs with benzedrine polycythemia, and one ephedrine-fed dog.

Aminophylline, given orally in a daily dose of 100 mg., definitely reduced the high erythrocyte numbers in 2 dogs having ephedrine-induced polycythemia, 2 dogs with amphetamine polycythemia, and 2 splenectomized rabbits in which polycythemia was induced by ephedrine and amphetamine.

Mannitol hexanitrate (30 mg. daily by stomach tube) partially depressed



cobalt polycythemia in 5 rabbits, and amphetamine polycythemia in one splenectomized dog.

Choline hydrochloride, in a daily dose of 100 mg. by stomach tube, was effective in partially reducing marked cobalt polycythemia in 3 rabbits.

The ingestion of whole liver (one-quarter pound daily) depressed ephedrine-induced polycythemia in one human subject, returning the red blood cell count to normal within 4 days, in spite of continued ephedrine administration.

The most plausible interpretation of the action of the foregoing substances is that they depress polycythemia probably by causing vasodilation of bone marrow arterioles, thus improving the blood supply to this tissue and diminishing the local anoxia which is probably the immediate stimulus to polycythemia.

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# STUDIES ON THE ACUTE TOXICITY OF SULFACETIMIDE (PARA-AMINOBENZENE SULFONYL ACETYL IMIDE)

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Received for publication July 7, 1941

Long, Haviland and Edwards (2) emphasized that peroral administration of certain poorly soluble derivatives of sulfanilamide is not a valid method of determining the acute toxicity of these compounds. Even when the soluble sodium salts are used, however, the toxicity values obtained by giving these drugs by mouth may vary considerably from those found by subcutaneous injection. This is well demonstrated by comparison of the findings of the above authors, those of van Dyke, Greep, Rake and McKee (3) and those of Barlow and Homburger (1). Such a comparison is made in table 1.

It is evident that the sodium salts (especially of the sulfathiazole derivatives) given by mouth would appear to be much less toxic than when they are injected parenterally. Hence results obtained by these two methods of administration may not be comparable, whether or not the compound involved is soluble. Long *et al.* (2) feel that with parenteral administration of the sodium derivatives "it is possible to determine with accuracy the true toxicity of the poorly soluble parent drug."

Extensive studies on the toxicity and absorption of sulfacetimide (para-aminobenzene sulfonyl acetyl imide) in the free acid form administered *per os* have been made by Harvey B. Haag (to be published elsewhere). In the light of the above criticism it was felt that data on the acute toxicity of the sodium salt of this compound when administered parenterally in a single dose would be of value. Preliminary experiments indicated that doses up to 7.5 grams per kilogram injected subcutaneously caused no deaths in mice. More exact studies were then conducted using doses larger than 7.5 grams per kilogram.

It is evident that if mice of various weights are injected with a standard volume of solution of given concentration, the dosage (per kilo body weight) will vary with the weight of the animal. Hence, if mice weighing between 22 and 24 grams are used, the dosage may vary from one mouse to the next by as much as 8 or 9 per cent. This is often the case when average weights of mice are used for calculating the dose to be administered.



In order to decrease this error, each animal, in the present studies, was weighed immediately before injection, and the volume of the dose was varied according to the weight. The concentration of the solution used was such that the volume required to administer a given amount of drug was never less

TABLE 1

*Acute toxicity of sodium salts of some sulfanilamide derivatives in mice. Peroral versus subcutaneous administration*

COMPOUND	ROUTE	DOSE <i>grams/ kilo</i>	PERCENT DEAD	AUTHOR
Sodium sulfanilamide. . . . .	Subcutaneous	3.0	44	Long, et al.
Sodium sulfapyridine. . . . .	Subcutaneous	1.0	50	Long, et al.
Sodium sulfathiazole. . . . .	Subcutaneous	0.95	50	van Dyke, et al.
" " "	Peroral	1.75	63.6	Barlow, et al.
" " "	Subcutaneous	1.95	50	Long, et al.
Sodium sulfathiazole . . . . .	Subcutaneous	1.45	50	van Dyke, et al.
Sodium sulfathiazole. . . . .	Peroral	7.0	54.5	Barlow, et al.
Sodium sulfaphenylthiazole. . . . .	Subcutaneous	0.9	50	Long, et al.
Sodium sulfaphenylthiazole. . . . .	Peroral	2.5	63.6	Barlow, et al.
Sodium sulfamethylthiazole . . . . .	Subcutaneous	0.86	50	Long, et al.
Sodium sulfamethylthiazole. . . . .	Peroral	6.0	63.6	Barlow, et al.
Sodium sulfacetimide. . . . .	Subcutaneous	9.7	45.5	Present work
Sodium sulfacetimide. . . . .	Subcutaneous	10.0	63.4	Present work
Sodium sulfadiazine. . . . .	Subcutaneous	1.5	43.5	Feinstone, et al.
Sodium sulfadiazine. . . . .	Subcutaneous	1.75	100.0	Feinstone, et al.

TABLE 2

#### Acute toxicity of sodium sulfacetimide in mice

GRAMS PER KILOGRAM	NUMBER OF NICE	MORTALITY IN 4 DAYS	PER CENT MORTALITY
7.5	20	0	0
8.0	53	11	20.8
8.5	40	9	22.5
8.8	40	9	22.5
9.0	31	9	29.0
9.5	28	10	35.7
9.7	44	20	45.5
10.0	41	26	63.4
10.2	12	9	75.0

than 0.35 cc. nor more than 0.55 cc. In this manner each animal actually receives the amount of drug recorded, within about 1 per cent.

It might appear at first that weighing each animal immediately before injection and calculating the volume of solution to be administered is a slow and tedious process. Actually, it can be done very quickly. A simple equation



showing the relationship between volume of dose, weight of animal and the concentration of the solution is set up. Since the concentration of the solution is constant for any given group of mice, the volume used is directly proportional to the weight of the animal. For example, at 9.5 grams per kilogram,

Let  $C$  = concentration of sodium sulfacetimide in solution

$V$  = volume of solution to be injected

$W$  = weight of mouse

$$\text{Then } V = \frac{9.5W}{1000C} = \frac{9.5W}{500} = 0.019W$$

By use of the slide rule the calculations go very rapidly.

As shown in the above table, the  $LD_{50}$  of sodium sulfacetimide for mice when injected subcutaneously lies between 9.7 and 10.0 grams per kilogram. According to Long *et al.* (2) the  $LD_{50}$  for sodium sulfapyridine is 1.0 gram per kilogram, for sodium sulfathiazole 1.95 grams per kilogram, for sodium sulfamethylthiazole 0.86 gram per kilogram, for sodium sulfaphenylthiazole 0.9 gram per kilogram and for sodium sulfanilamide about 2.92 grams per kilogram. Feinstone, *et al.* (4) report to  $LD_{50}$  of sodium sulfadiazine to be between 1.5 and 1.75 grams per kilogram. Even when sodium sulfacetimide administered by the subcutaneous route is compared with sodium sulfapyridine or the sodium sulfathiazole derivatives administered perorally (*vide* Barlow and Homburger (1)) it is apparent that the sulfacetimide compound is by far the least toxic.

#### SUMMARY

Mice tolerate single doses as high as 7.5 grams of sodium sulfacetimide per kilogram body weight when administered subcutaneously. The  $LD_{50}$  of this compound lies between 9.7 and 10.0 grams per kilogram.

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# THE THERAPEUTIC EFFECT OF SULFACETIMIDE IN EXPERIMENTAL INFECTIONS DUE TO ESCHERICHIA COLI

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Received for publication July 7, 1941

The frequency with which *Escherichia coli* is the causative agent of urinary tract infections has led to considerable clinical use of various chemotherapeutic agents in such cases. In contrast to this are the relatively few studies which have been made of the efficacy of chemotherapy in experimental *E. coli* infections. The purpose of the present work is to determine the therapeutic value of sulfacetimide (*p*-aminobenzene sulfonyl acetyl imide) in experimental peritonitis caused by this organism in mice.

Long and Bliss (1939) stated that sulfanilamide is without effect in such infections. These findings are contradicted by the work of Cooper, Gross and Lewis (1939) who showed that 20 mg. of sulfanilamide daily administered per os saved 80 per cent of the mice infected intra-abdominally with as many as 100 lethal doses of this organism. Assuming that the mice averaged 20 grams in weight, the daily dose of the drug was about 1 gram per kilogram. Using a strain of *E. coli* isolated from a case of pyelonephritis, Kolmer and Rule (1939) found that 0.160 gram of sulfanilamide or sulfapyridine per kilogram administered twice daily by subcutaneous or intra-abdominal injection did not save mice infected intra-abdominally with 1 to 2 M.L.D.

It is not possible to make a direct comparison of the virulence of the strains of *E. coli* employed above. One M.L.D. of that used by Kolmer and Rule was about 0.2 cc. of a 24-hour broth culture of the organism. The virulence of each of the two strains used by Cooper, *et al.* was enhanced by diluting the cultures with 5 per cent mucin. One M.L.D. of one strain thus treated was 0.5 cc. of a 1:1000 dilution of a six hour culture while a second strain was about 10 times as virulent. The approximate number of cells per M.L.D. was not indicated in either of the above studies. In the present work it was found preferable to indicate the M.L.D. in terms of numbers of cells rather than in terms of dilution.

The organism which we employed was recently isolated from a urinary tract infection. Brief studies indicated that 500,000 cells from a 15- to 18-hour broth culture diluted with 5 per cent mucin killed 20-25 gram male, swiss



mice in less than 24 hours following intra-abdominal injection. An occasional mouse, however, was found to be resistant to even 5 times this dose. Consequently, the M.L.D. here is considered to be that number of cells suspended in mucin which kills 95 per cent of the mice in 18 to 24 hours. With this criterion it has been possible to obtain very consistent results.

## METHODS

Male, swiss mice, 22-25 gram, were infected by intra-abdominal injection of 0.1 cc. of a 5 per cent mucin suspension containing known numbers of cells from an 18 hour broth culture. (The number of cells per cubic centimeter in the broth culture before dilution was determined by counting in a haemocytometer chamber.)

TABLE 1

*Therapeutic effects of sulfacetimide and sulfanilamide in experimental Esch. coli infections in mice*

DRUG	DOSE	INOCULUM NUMBER CELLS	M.L.D.	NUMBER OF MICE	DEATHS—DAYS AFTER INFECTION										SURVIVALS	
					1	2	3	4	5	6	7	7-14	Number	Per cent		
	<i>grams/kilo b.i.d.</i>															
Sulfacetimide.....	1.0	$1 \times 10^6$	2	14									14	100		
Sulfanilamide.....	1.0	$1 \times 10^6$	2	10				2				1	7	70		
Controls.....		$1 \times 10^6$	2	14	14								0	0		
Sulfacetimide.....	0.5	$2.5 \times 10^6$	5	40	14	9	1	1					15	37.5		
Sulfanilamide.....	0.5	$2.5 \times 10^6$	5	20	15	1		1					3	15		
Controls.....		$2.5 \times 10^6$	5	40	39								1	2.5		

For purposes of comparison some animals were treated with sulfanilamide while others received sulfacetimide. The drugs were suspended in 15 per cent gum acacia and administered per os. The first dose was given within an hour after the infection, the second dose 5 hours thereafter, and the remaining doses twice daily for 4 additional days. Heart blood cultures were made from all animals which died during the experiment and were found invariably to contain *Escherichia coli*.

## RESULTS

As shown in table 1, one gram of sulfacetimide per kilo twice daily saved 100 per cent of the mice inoculated with one million cells of *Escherichia coli*. Sulfanilamide at the same dosage saved 70 per cent of the animals.

When the animals were inoculated with two-and-one-half million cells and given only 0.5 gram of the drugs per kilo twice daily, sulfacetimide saved 37.5 per cent of the animals while sulfanilamide saved 15 per cent.

## SUMMARY

On the basis of equal oral dosage sulfacetimide produced higher survival rates than sulfanilamide in mice experimentally infected with *Escherichia coli*.



However, since the blood concentration-time curves obtained with the dosage schedule employed may be dissimilar for the two drugs, it cannot be presumed from these experiments that sulfacetimide is more active therapeutically than sulfanilamide in this infection.

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# THE TOXICITY AND EFFECT OF CERTAIN FLUORINATED DERIVATIVES OF PHENYLALANINE AND TYROSINE ON THE BASAL METABOLIC RATE OF THE RAT<sup>1</sup>

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Received for publication July 10, 1941

Numerous investigations have been conducted on the possible relationship of fluorine to thyroid activity, but there has been disagreement as to how the gland is affected. Chang, Phillips and Hart (2) found a large increase in the fluorine content of the thyroid gland of cows fed fluorine-containing rock phosphate. Goldemberg (8) reported that sodium fluoride caused a lowering of the basal metabolic rate (B.M.R.) in rats, and Goldemberg (8), Gorlitzer (10) and Schutte (26) have made use of fluorides in the treatment of human hyperthyroidism (9). Orlowski (20) reported that fluorine improved the subjective symptoms of hyperthyroidism, but that a real cure of the disorder could not be obtained. In contrast to Goldemberg's results Phillips and co-workers (21, 22) showed that sodium fluoride did not lower the B.M.R. of normal rats and, in addition, demonstrated that fluorine definitely increased the toxicity of desiccated thyroid for the guinea pig, rat, and chick (22, 23). Evans and Phillips (5) found no relationship between the fluorine content of the thyroid gland and the degree of hyperthyroidism in human patients. Seevers and Braun (28) showed that fluorine did not retard the effect of desiccated thyroid on the rabbit and Stormont, Kozelka, and Seevers (29) demonstrated that fluorine did not cause an iodine deficiency in the rabbit. Schteingart and Sammartino (25) have reported no benefit from the use of sodium fluoride in treating hyperthyroidism.

The synthesis of 3-fluorotyrosine by Schiemann and Winkelmüller (24) was followed by the work of Kraft (12), who reported that this compound antagonized the effect of thyroxine on tadpoles, and of Litzka (15), who reported that 3-fluorotyrosine acted antagonistically to thyroid secretions. Litzka (15), Kraft and May (13), and May (17) have reported favorable results from

<sup>1</sup> Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. These studies were supported in part by funds from the Wisconsin Alumni Research Foundation and by the Moorman Manufacturing Company, Quincy, Illinois, and the Hoover and Mason Phosphate Company of Tennessee. We are also indebted to the Works Progress Administration Project No. 10305 for aid in the fluorine analyses.



the use of 3-fluorotyrosine in treating hyperthyroidism. More recently English, Mead and Niemann (4) have reported an improved procedure for the preparation of 3-fluorotyrosine and in addition have prepared 3-fluorophenylalanine, 3-fluoro-5-iodotyrosine, and 3,5-difluorotyrosine (4).<sup>2</sup> The study of these compounds is of particular interest because of their close structural relationship to thyroxine and its postulated precursors. These investigations were undertaken to study the toxic symptoms, determine the lethal dose, and to ascertain if these fluorinated compounds affect the basal metabolic rate of the rat.

## EXPERIMENTAL PROCEDURES AND RESULTS

### *I. Toxicity studies*

The acute toxicity of the fluorinated compounds for rats was determined by giving single subcutaneous injections after dissolving the compound in a minimum amount of hydrochloric acid. The levels injected, and the data obtained on the acute toxicity of these compounds when given to mature animals weighing 200 grams or more are summarized in table 1.

The minimum lethal dose was considered to be that dose which would result in death of 50 per cent of the animals to which it was given. On this basis, the M.L.D. for 3-fluorotyrosine was found to be approximately 12.5 mg. per kilogram of body weight; for 3-fluorophenylalanine 20 mg. per kilogram; for 3-fluoro-5-iodotyrosine 65 mg. per kilogram; and for 3,5-difluorotyrosine slightly greater than 40 mg. per kilogram. The latter compound was not available in sufficient amounts to permit us to administer higher doses. When given to rats weighing less than 100 grams the compounds were more toxic: the minimal non-lethal dose was found to be 6, 12.5, and 15 mg. per kilogram for 3-fluorotyrosine, 3-fluorophenylalanine, and 3-fluoro-5-iodotyrosine respectively, whereas for mature animals the corresponding doses were 10, 15, and 40 mg. per kilogram.

The symptoms of toxicity were similar for all of the compounds studied, and therefore need to be discussed in detail for 3-fluorotyrosine only. Alternate periods of depression and hyperirritability became evident in the rat several hours after injection of the M.L.D. of 3-fluorotyrosine. This was soon followed by convulsive seizures, similar to those of hypocalcemia. These were marked by early twitching, which increased in intensity, followed by tautness and uncontrollable swaying of the body. The seizures became more violent 8 to 12 hours after the injection. The rats dashed violently around the cage. Frequently they lay on their sides or backs while convulsive movements of the legs continued. Sometimes the rats rose upright, with the entire body in

<sup>2</sup>These compounds were made available to us for this investigation by Dr. Carl Niemann, California Institute of Technology, with whom this is a cooperative research project.



tremors, then fell backwards or sideways. Frequent voiding of the urine occurred. During the convulsions the rats often ran into the side of the cage, or bit their mouths and lips so that hemorrhage occurred.

Between convulsive seizures the rats were totally limp and extremely lethargic. During these periods the rats were difficult to rouse, but moved if probed sharply, indicating a response to pain. Breathing in the lethargic state was rapid and shallow while the pulse rate was rapid. Death usually occurred during the period of depression. The occurrence of convulsions and periods of depression was not always followed by death, as a number of rats recovered after exhibiting these symptoms.

The toxic symptoms of 3-fluorotyrosine poisoning were somewhat more violent than for the other compounds. Convulsions were less frequent with

TABLE 1  
*Acute toxicity of certain organic fluorine compounds*

3-FLUOROTYROSINE			3-FLUOROPHENYL- ALANINE			3-FLUORO-5-IODOTYRO- SINE			3,5-DIFLUOROTYROSINE		
Dose	Number of rats	Number died	Dose	Number of rats	Number died	Dose	Number of rats	Number died	Dose	Number of rats	Number died
mg./kg.			mg./kg.			mg./kg.			mg./kg.		
5	1	0	10	2	0	10	2	0	5	1	0
6	1	0	12.5	1	0	20	15	0	10	2	0
10	10	0	15	2	0	30	8	0	15	1	0
12	7	1	20	17	8	35	5	0	20	3	0
12.5	6	3	25	4	4	40	10	0	30	3	0
15.0	9	7	30	1	1	45	5	1	40	11	3
20.0	6	6				50	5	2			
						60	5	2			
						65	4	2			
						80	3	3			
						100	2	2			

3-fluorophenylalanine or 3-fluoro-5-iodotyrosine, and few were observed with 3,5-difluorotyrosine. When the latter compound was given the rats showed mainly the depressed, lethargic state. Convulsions in mature animals occurred on doses as low as 8, 15, and 30 mg. per kilogram for 3-fluorotyrosine, 3-fluorophenylalanine, and 3-fluoro-5-iodotyrosine respectively. When the dose was lethal, death occurred in most cases within 24 hours after administration of the compound, but death directly attributable to one of these compounds sometimes occurred as late as 3 days after the administration.

The toxicity of 3-fluorotyrosine when fed in the ration was studied by adding the compound to a low fluorine basal ration consisting of yellow corn 76, reprecipitated casein 15, yeast 3, cod liver oil 1, sodium chloride 1, dicalcium phosphate 3, and starch 1. The starch served as a carrier for the small amounts of 3-fluorotyrosine added.



Eight groups of weanling rats were fed *3*-fluorotyrosine for a four-week period as indicated in table 2. The two levels of sodium fluoride were fed as controls. The 0.0165 per cent level of sodium fluoride supplied the same amount of fluorine as the 0.075 per cent level of *3*-fluorotyrosine. The 0.1 per cent level of sodium fluoride is a level which is known to definitely inhibit growth (14). The effects of the levels of *3*-fluorotyrosine on survival, gain in weight, food consumption and fluorine deposition in the bone are summarized in table 2. All the rats receiving the 0.025 per cent, 0.05 per cent and 0.075 per cent level of *3*-fluorotyrosine died in from 1 to 3 days after being put on experiment. Death occurred on the 0.005 per cent level from 1 to 12 days, and in the 0.004 per cent level from 2 to 22 days. The rats which died showed symptoms similar to those discussed previously for acute *3*-fluorotyrosine toxicity.

TABLE 2

*Effect of feeding 3-fluorotyrosine to growing rats (4-week period)*

LEVEL FED	NUMBER OF RATS	NUMBER DIED	AVERAGE WEEKLY WEIGHT GAIN	AVERAGE DAILY FOOD CONSUMPTION	AVERAGE FLUORINE IN BONES
<i>per cent</i>			<i>grams</i>	<i>grams</i>	<i>p.p.m.</i>
None added	4	0	30	10.1	606
0.0005	4	0	23	8.4	133
0.001	4	0	22	7.3	199
0.0025	5	1	20	6.9	229
0.004	7	3	18	6.0	109
0.005	10	7	18	6.1	
0.025	4	4	Lethal		
0.05	3	3	Lethal		
0.075	3	3	Lethal		
0.0165% NaF	4	0	29	9.1	1,288
0.1% NaF	4	0	19	7.7	5,502

*3*-Fluorotyrosine at all levels fed in these experiments retarded the growth approximately the same as 0.1 per cent sodium fluoride. In contrast, the 0.0165 per cent level of sodium fluoride, which furnished 10 times as much fluorine as the 0.005 per cent level of *3*-fluorotyrosine and 100 times as much fluorine as the 0.0005 per cent level, had no effect on growth. This clearly indicated that the toxicity of *3*-fluorotyrosine was not due to liberation of inorganic fluorides. The bone analyses supplied further evidence that inorganic fluoride was not liberated, since the bones of the rats receiving *3*-fluorotyrosine contained less fluorine than those of the controls, and both the levels of sodium fluoride fed caused marked skeletal fluorine deposition. The fluorine analyses on the bones were made according to a method previously used in this laboratory (6).

## II. Basal metabolism studies

A basal metabolism apparatus patterned after that described by Schwabe and Griffith (27) was constructed for the B.M.R. determinations. The ap-



29–30°C., as recommended by Forbes and Swift (7). Rats upon which determinations were to be made were placed without access to food in the constant temperature room 28 to 32 hours before the B.M.R. was determined, as suggested by Benedict and MacLeod (1). Food intake was standardized by giving each rat 2.5 grams of ration, an amount which they readily consumed, 20 to 24 hours before the determinations. B.M.R. determinations were made at weekly intervals. Between determinations rats were maintained on the

TABLE 3

*Effect of 3-fluorotyrosine, 3-fluorophenylalanine and 3-fluoro-5-iodotyrosine on the B.M.R. of the rat when fed in the ration*

WEEK	LEVEL IN RATION	CALORIES PER SQ. M. PER 24 HOURS		LEVEL IN RATION	CALORIES PER SQ. M. PER 24 HOURS	
		Rat 1	Rat 2		Rat 3	Rat 4
0	<i>per cent</i> Normal B.M.R.	906	952	<i>per cent</i> Normal B.M.R.	1,006	989
3-fluorotyrosine administration						
1	0.0005	937	974	0.001	991	
2	0.001	888	948	0.001	1,070	1,078
3	0.002	932	970	0.001	1,034	973
5	Rechecked B.M.R.	853	961	Rechecked B.M.R.	975	942
3-fluorophenylalanine administration						
6	0.001	897	1,021	0.002	1,070	1,060
7	0.002	987	967	0.002	1,069	926
8	0.004	870	918	0.002	1,063	1,016
10	Rechecked B.M.R.	928	909	Rechecked B.M.R.	995	1,067
3-fluoro-5-iodotyrosine administration						
11	0.001	966	843	0.002	902	1,036
12	0.002	815	889	0.002	814	854
13	0.004	762	826	0.002	833	873

regular stock ration used in this laboratory, and the fluorinated compounds administered as indicated below.

Before the compounds studied were administered the basal metabolic rate of all the rats was determined at weekly intervals until a satisfactory basal level for each animal had been established. The B.M.R. was expressed in terms of calories per square meter of body surface per 24 hours, the surface area formula of Diack (3) being used for the calculations. Variations in the B.M.R. did not exceed 10 per cent in the normal animal when standardized



under these experimental conditions, and usually the B.M.R. did not vary more than 5 or 6 per cent. In these experiments the B.M.R. was not considered to be affected unless the variation exceeded 10 per cent.

Eight mature male rats were used for the first series of studies. Four rats were fed *β*-fluorotyrosine: 2 received 0.001 per cent in the ration for 3 weeks; and 2 received 0.0005 per cent for the first week, 0.001 per cent for the second

TABLE 4

*Effect of β-fluorotyrosine, β-fluorophenylalanine, and β-fluoro-δ-iodotyrosine on the B.M.R. of the rat when given by subcutaneous injection*

WEEK	LEVEL INJECTED	CALORIES PER SQ. M. PER 24 HOURS		LEVEL INJECTED	CALORIES PER SQ. M. PER 24 HOURS	
		Rat 5	Rat 6		Rat 7	Rat 8
	mg./kg.			mg./kg.		
0	Normal B.M.R.	868	960	Normal B.M.R.	1,034	943
<i>β</i> -fluorotyrosine administration						
1	1.25	915	1,042	2.50	1,118	871
2	2.50	803	1,074	2.50	951	926
3	5.00	952	995	2.50	1,040	924
5	Rechecked B.M.R.	998*	957	Rechecked B.M.R.	1,038	944
<i>β</i> -fluorophenylalanine administration						
6	2.50	960		5.00	1,060	1,058
7	5.00	900	1,003	5.00	1,080	962
8	10.00	993	875	5.00	993	895
10	Rechecked B.M.R.	981	946	Rechecked B.M.R.	994	893
<i>β</i> -fluoro-δ-iodotyrosine administration						
11	2.50	959	1,033	5.00	1,006	885
12	5.00	962	857	5.00	921	819
13	10.00	961	1,040	5.00	933	976

\* A different rat was used for the remainder of the studies. The value is its normal B.M.R.

week, and 0.002 per cent the third week. These levels were based on the study of the toxicity of the compound when fed in the ration, and were all equal to or greater than the lowest level shown to effect growth. Food-consumption records were kept in all feeding experiments.

Four rats received *β*-fluorotyrosine by injection: two received 2.5 mg. per kilogram for each of three weeks; and two received 1.25 mg. per kilogram the first week, 2.50 mg. per kilogram the second week, and 5.00 mg. per kilogram



the third week. Injections were usually made 8 hours before the B.M.R. was determined, but the time of injection was varied from 6 to 24 hours to ascertain if there was any difference in the effect produced. No difference was noted.

The same animals were used for the study of *3*-fluorophenylalanine, and *3*-fluoro-*5*-tyrosine. A period of 2 weeks between tests was allowed, and during this period the B.M.R. was checked for agreement with the established basal. The effects of *3*-fluorophenylalanine and *3*-fluoro-*5*-iodotyrosine were ascer-

TABLE 5  
*Effect of thyroxin on rats given 3-fluoro-5-iodotyrosine*

WEEK	RAT 17		RAT 18	
	Treatment	Calories per sq. m. per 24 hours	Treatment	Calories per sq. m. per 24 hours
<i>3</i> -fluoro- <i>5</i> -iodotyrosine in the ration				
0	(1) None, normal B.M.R.	1,020	(1) None, normal B.M.R.	1,061
1	(2) 0.02 per cent <i>3</i> -fluoro- <i>5</i> -iodotyrosine in ration	923	(2) 0.02 per cent <i>3</i> -fluoro- <i>5</i> -tyrosine in ration	1,150
2	(3) Same as (2) + thyroxin, 1 mg./kg. 48 hrs. previous	1,381	(3) Same as (2)	1,159
3	(4) Same as (3)	1,277	(4) Same as (2) + thyroxin, 1 mg./kg. 48 hrs. previous	1,646
<i>3</i> -fluoro- <i>5</i> -iodotyrosine by injection				
	RAT 19		RAT 20	
	Treatment	Calories per sq. m. per 24 hours	Treatment	Calories per sq. m. per 24 hours
0	(1) None, normal B.M.R.	1,025	(1) None, normal B.M.R.	1,191
1	(2) Thyroxin, 1 mg./kg. 48 hrs. previous. <i>3</i> -fluoro- <i>5</i> -iodotyrosine, 50 mg./kg. 24 hrs. previous	1,038	(2) <i>3</i> -fluoro- <i>5</i> -iodotyrosine, 50 mg./kg. 24 hrs. previous	1,087
2	(3) Same as (2)	1,398	(3) Same as (2) + thyroxin, 1 mg./kg.	Rat died

tained by determination of the B.M.R., using the same experimental routine as used for *3*-fluorotyrosine, except that the levels administered in the ration and by injection were twice as large.

The results of these studies are summarized in tables 3 and 4. The data showed that *3*-fluorotyrosine and *3*-fluorophenylalanine were entirely without effect on the B.M.R. when fed or injected. However, *3*-fluoro-*5*-iodotyrosine appeared to lower the B.M.R. slightly more than 10 per cent when the compound was fed in the ration. In order to recheck any possible effect of the



3-fluoro-5-iodotyrosine, the compound was given at higher levels both by injection and in the ration in conjunction with thyroxin, as indicated in table 5. On the basis of food consumption the 0.02 per cent level in the ration supplied 3-fluoro-5-iodotyrosine at a rate of approximately 50 mg. per kilogram of body weight per week. The results show that the high levels had no, or at best, a slight depressing effect on the B.M.R., and, more important, that the compound did not prevent the increase in the B.M.R. resulting from thyroxin injections. The administration of 3,5-difluorotyrosine (table 6) had no effect on the B.M.R. at the levels fed. However, when it was given at the level of 30 mg. per kilogram approximately three-fourths of the M.L.D., there was an average lowering of the B.M.R. of 15 per cent, accompanied by toxic symptoms.

The effect of these compounds on liver glycogen was also studied. Six groups of 10 rats each were fed for a period of 2 to 5 weeks as follows: Lot I, basal ration only; Lot II, basal plus 0.001 per cent 3-fluorotyrosine; Lot III,

TABLE 6  
*Effect of 3,5-difluorotyrosine in the B.M.R. of the rat*

WEEK	FED IN RATION			SUBCUTANEOUS INJECTION		
	Level fed	Calories per sq. m. per 24 hours		Level injected	Calories per sq. m. per 24 hours	
		Rat 9	Rat 10		Rat 11	Rat 12
	<i>per cent</i>			<i>mg./kg.</i>		
0	Normal B.M.R.	981	1,112	Normal B.M.R.	1,100	1,002
1	0.004	985		10.0		1,001
2	0.008	945	1,110	20.0	1,210	1,003
3	0.016	963	1,261	30.0	880	904

basal plus 0.002 per cent 3-fluorophenylalanine; Lot IV, basal plus 0.002 per cent 3-fluoro-5-iodotyrosine; Lot V, basal plus 0.1 per cent sodium fluoride; and Lot VI, basal plus 0.25 per cent desiccated thyroid. None of the groups fed any of the fluorinated compounds showed a change in liver glycogen as compared with the basal. The group fed desiccated thyroid showed a marked drop in liver glycogen to an average of less than one-third of the basal group.

#### DISCUSSION

The symptoms of 3-fluorotyrosine toxicity and the minimal lethal dose agree with those reported by Litzka (15, 16). It should be noted, however, that Litzka claimed that no ill effects followed the daily administration of two-thirds of the toxic dose of 3-fluorotyrosine. Our results indicate that in young rats levels as small as one-tenth the lethal level in the ration cause a definite inhibition of growth.

The compounds studied are decidedly more toxic than inorganic fluoride.



the third week. Injections were usually made 8 hours before the B.M.R. was determined, but the time of injection was varied from 6 to 24 hours to ascertain if there was any difference in the effect produced. No difference was noted.

The same animals were used for the study of 3-fluorophenylalanine, and 3-fluoro-5-tyrosine. A period of 2 weeks between tests was allowed, and during this period the B.M.R. was checked for agreement with the established basal. The effects of 3-fluorophenylalanine and 3-fluoro-5-iodotyrosine were ascer-

TABLE 5  
*Effect of thyroxin on rats given 3-fluoro-5-iodotyrosine*

WEEK	RAT 17		RAT 18	
	Treatment	Calories per sq. m. per 24 hours	Treatment	Calories per sq. m. per 24 hours
<i>3-fluoro-5-iodotyrosine in the ration</i>				
0	(1) None, normal B.M.R.	1,020	(1) None, normal B.M.R.	1,061
1	(2) 0.02 per cent 3-fluoro-5-iodotyrosine in ration	923	(2) 0.02 per cent 3-fluoro-5-tyrosine in ration	1,150
2	(3) Same as (2) + thyroxin, 1 mg./kg. 48 hrs. previous	1,381	(3) Same as (2)	1,159
3	(4) Same as (3)	1,277	(4) Same as (2) + thyroxin, 1 mg./kg. 48 hrs. previous	1,646
<i>3-fluoro-5-iodotyrosine by injection</i>				
	RAT 19		RAT 20	
	Treatment	Calories per sq. m. per 24 hours	Treatment	Calories per sq. m. per 24 hours
0	(1) None, normal B.M.R.	1,025	(1) None, normal B.M.R.	1,191
1	(2) Thyroxin, 1 mg./kg. 48 hrs. previous. 3-fluoro-5-iodotyrosine, 50 mg./kg. 24 hrs. previous	1,038	(2) 3-fluoro-5-iodotyrosine, 50 mg./kg. 24 hrs. previous	1,087
2	(3) Same as (2)	1,398	(3) Same as (2) + thyroxin, 1 mg./kg.	Rat died

tained by determination of the B.M.R., using the same experimental routine as used for 3-fluorotyrosine, except that the levels administered in the ration and by injection were twice as large.

The results of these studies are summarized in tables 3 and 4. The data showed that 3-fluorotyrosine and 3-fluorophenylalanine were entirely without effect on the B.M.R. when fed or injected. However, 3-fluoro-5-iodotyrosine appeared to lower the B.M.R. slightly more than 10 per cent when the compound was fed in the ration. In order to recheck any possible effect of the



iodotyrosine, and slightly more than 40 mg. per kilogram for *3,5*-difluorotyrosine. The acute toxic effects were similar for the four compounds and were characterized by periods of convulsion and hyperirritability followed by extreme lethargy.

When fed in the ration at levels as low as 0.0005 per cent *3*-fluorotyrosine inhibited growth of young rats; 0.0025 per cent in the ration caused an inhibition of growth equivalent to that of 0.1 per cent sodium fluoride. Levels of 0.004 to 0.005 per cent fed in the ration were lethal.

The *3*-fluorotyrosine and the *3*-fluorophenylalanine had no effect on the B.M.R. of the rat, when given in amounts up to one-half of the M.L.D., *3,5*-difluorotyrosine and *3*-fluoro-*5*-iodotyrosine likewise had little or no effect on the B.M.R. and the latter did not prevent a calorogenic response to thyroxine. If the former was given in doses of approximately three-fourths of the M.L.D., a very slight drop in the B.M.R. was obtained.

The highly toxic nature of the compounds studied contra-indicates their use as therapeutic agents for the control of the B.M.R. in the rat under experimental conditions.

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A STUDY OF THE ACQUIRED RESISTANCE OF FIXED TISSUE  
CELLS MORPHOLOGICALLY ALTERED THROUGH  
PROCESSES OF REPAIR<sup>1</sup>

V. CONCERNING PHYSICAL MODIFICATIONS OF CELLS ASSO-  
CIATED WITH THE DEVELOPMENT OF A RESISTANCE TO  
CERTAIN CHEMICAL AGENTS. THE ACQUIRED RESIST-  
ANCE OF THE KIDNEY TO BICHLORIDE OF MERCURY

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Received for publication July 17, 1941

For a number of years the major interest in this laboratory has been an attempt to induce cell changes of such a degenerative order by the use of various chemical substances that when processes of repair were established and completed in such tissue, they would lead to the formation of cells of a modified or altered morphology for the tissue location under consideration. By the use of uranium as a toxic agent for both the kidney and the liver, when the ages (1), (2), of the animals are taken into consideration, it has been possible to induce in these organs an acute injury of such an order that when tissue repair was effected the process resulted in the formation of epithelial tissue of a changed morphological type. Such cells were not only resistant to the cause of the acute injury, uranium nitrate, which provoked the repair process, but they were also resistant to bodies of an entirely different chemical character, such as chloroform and ether (3), (4), (5), (6).

The earlier experimental studies of von Mehring (7), Heineke (8) and Kaufmann (9) of the mode of action of bichloride of mercury as a nephrotoxic and hepatotoxic agent considered this influence to be in large measure due to the ability of this metallic salt to induce changes in the blood vessels, especially characterized by thrombus formation, while the somewhat later studies of Schmeideberg (10) and of Burmeister and McNally (11) indicated a dominant epithelial injury in such intoxications. This latter concept has been confirmed by work from this laboratory (12), (13) and furthermore, in these experiments in which dogs were used the observation was made that the localization of the epithelial injury in the kidney was at first confined to the proximal convoluted segment of the nephron. This is also the segment at which uranium exerts its selective toxic influence.

<sup>1</sup> This investigation was made possible by a grant from the Josiah Macy, Jr. Foundation.



In a recent preliminary communication (14) observations have been recorded which indicate that, if the proximal convoluted segment of the nephron is sufficiently injured by uranium nitrate, in those animals that survive the epithelial repair in this location of the tubule takes place by the formation of an abnormal and a typical type of cell or syncytial structure which in turn is resistant to toxic amounts of bichloride of mercury. The present study represents a continuation of these initial experiments.

#### METHODS

Thirty-eight middle age dogs, four to six years old, were employed in these experiments. The animals were subjected to the usual studies of both the blood and urine in order to ascertain if they represented normal experimental material and to exclude a naturally acquired nephritis (15). Four of these animals were used in control experiments. They were given 10 mgm. of morphine sulfate per kilogram subcutaneously in order to empty the stomach and, by its narcotic action, to facilitate retention of the bichloride given subsequently. While under the influence of morphine all of the animals were given 15 mgm. of bichloride of mercury per kilogram by stomach tube. Both the blood and the urine were examined for the presence of mercury.

The four control dogs have in general followed the same course both clinically and pathologically as those animals discussed in a previous publication (13). One of them died on the third day, the remaining three succumbed on the eighth day of the bichloride intoxication. The changes in both the kidney and the liver were characteristic for the bichloride injury. Both the time of appearance and the severity of the renal injury were related to the intensity of the reaction for mercury in the blood and urine. There was no histological evidence of either normal or atypical epithelial repair in these organs (fig. 1).

The remaining 34 animals were first given subcutaneously 4 mgs. of uranium nitrate per kilogram. For dogs of this age period, four to six years, such an amount of this substance approaches the lethal dose. Of the 34 animals, 26 survived the uranium injury. Their course during this period of injury was of the order previously described (15) (fig. 2). The urine and blood gave evidence of an acute, severe nephritis which finally resulted in the development of a chronic injury, as indicated by the appearance in the urine of a small amount of albumin, broad, finely granular and hyaline casts, and a small number of red blood cells. There was some reduction in both the time of appearance and elimination of phenolsulphonephthalein and a moderate retention of urea and nonprotein nitrogen. There was no retention of creatinin. The time necessary for such a degree of chronic renal injury to develop was variable, depending on the severity of the acute injury. Seven weeks to 4½ months would embrace the recovery period for all of the animals. Biopsy material removed from the kidneys of such dogs has shown in general very marked changes in the glomeruli and in the cellular morphology of the epithelium lining, especially the proximal convolution of the nephron. The glomeruli have shown both a periglomerular and intracapillary fibrosis, the latter resulting in a variable degree of glomerular capillary obliteration. The epithelium lining the proximal convoluted tubule has changed entirely in its architecture as a result of the acute uranium injury followed by a process of repair. These cells are no longer cuboidal in configuration with a brush border and with large, rather faintly staining, spherical nuclei, but have been replaced by a flattened, atypical type of epithelial cell, or by an epithelial tissue which frequently fails to show cell differentiation, but exists as syncytial structures in part relining the repaired tubular segments. The cytoplasm of such cells and syncytia stains intensely and gives one the impression of cytoplasmic denseness.



The nuclei are usually oblong or ovoid, large in proportion to the surrounding cytoplasm and stain deeply, usually failing to show chromatic granules.

The 26 animals having developed the type of kidney repair which has been indicated above were now available for a study of the toxic effect of bichloride of mercury as expressed by its ability to effect an injury to that segment of the renal nephron, the proximal convolution, for which in normal dogs it has a selective affinity. These dogs were subjected to the same experimental technique employed for the four control bichloride animals. The blood and urine were tested for the presence of mercury before the intoxications and daily for 14 days after the intoxication, and none was found. The qualitative test developed by Elliott (16) has been used for this purpose. The test in terms of the intensity of the reaction is of some quantitative value. The animals were then given 10 mgm. of morphine sulfate per kilogram subcutaneously and after the development of the narcotic effect were given by stomach tube 15 mgm. per kilogram of bichloride of mercury. None of this solution was regurgitated by the animals over a period of three and a half hours. Nine of the 26 dogs developed a gastroenteritis sufficiently intense to lead to dehydration and shock and seven of these died within forty-eight hours.

### RESULTS

Nineteen dogs survived the bichloride intoxication. In these animals the course of the poisoning showed variations in the severity and duration of the gastroenteritis and in other manifestations. When the gastroenteritis was severe, with a marked loss of fluid, the daily output of urine was reduced below the established normal value. In other animals in which the gastroenteritis was less severe, or failed to develop, there was evidence of a mercurial diuresis. The amount of urine was increased to as much as 410 cc. over the normal daily output. Both the blood and the urine, with two exceptions, contained mercury during the 2 weeks the animals were under observation. In the two exceptions referred to, the blood showed a trace of mercury without it being detectable in the urine. From qualitative observations there appeared to be a definite relationship between the blood and the urine mercury. This reached its height in both the blood and the urine between the fourth and seventh day of the intoxication, after which there was a decline in the intensity of the test. It should be noted that it is at such a period, fourth to the tenth day, that the renal injury in normal dogs is of the most advanced acute order. The urine of these animals contained an amount of albumin which varied from a trace to 2 grams per liter. Nine of the dogs after the subsidence of the gastroenteritis failed to show any reduction from the normal percentage elimination of phenolsulphonephthalein. In the remaining 10, the output of this dye was reduced, the maximum reduction being to 51 per cent in a two-hour period in one of the animals. Twelve of the animals showed a retention of nonprotein nitrogen which in the dog with the maximum decrease in the elimination of phenolsulphonephthalein reached 84 mg. per 100 cc. of blood.

These 19 animals which had survived an acute uranium intoxication, with, as shown by a study of biopsy material, an atypical type of repair process having developed in the kidney, and which had furthermore survived an acute mercuric chloride intoxication, were either autopsied or biopsy material was ob-





FIG. 1. COLORED MICROPHOTOGRAPH, ZEISS,  $\times 600$

The microphotograph is from the control dog kidney of experiment 4 that received 15 mgm. of bichloride of mercury by stomach tube. The animal survived a severe acute gastroenteritis and died on the 8th day of the mercury intoxication in a state of anuria. The figure shows in its center a glomerulus with an intact Bowman's membrane. The glomerular capillaries are distended with blood and in one area near the base of the structure there has occurred an apparent dissolution of several capillary loops. In the region of the base of the glomerulus are four tubules, descending and ascending limbs of Henle's loop in which the epithelium remains uninjured. In other areas surrounding the glomerulus are the proximal convolutions of the nephron showing the characteristic and localized toxic effect of bichloride of mercury. These changes are indicated by various stages in epithelial necrosis which in one of the segments of the nephron has resulted in a disappearance of the epithelial tissue. The basement membrane of this segment remains.







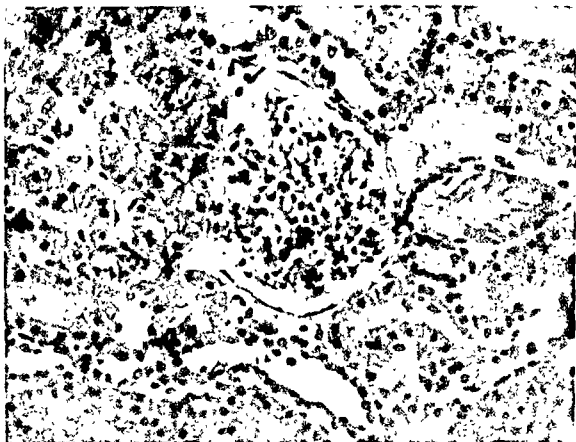


FIG. 2. COLORED MICROPHOTOGRAPH, ZEISS,  $\times 600$

The microphotograph is from the kidney of the animal of experiment 12. This dog was intoxicated by the subcutaneous injection of 4 mgm. of uranium nitrate. The animal, as the figure indicates, developed a severe acute renal injury. The figure is made from biopsy material obtained from the kidney on the sixth day of the uranium intoxication. The glomerular capillaries are distended with blood and fill the sub-capsular space. These structures have not undergone dissolution. Bowman's membrane is intact. At the base and at that part of the glomerulus distal to the base are shown segments of the loops of Henle in which the epithelium has failed to participate in the acute uranium injury. In other areas surrounding the glomerulus are segments of the proximal convolution of the nephron which show through processes of degeneration the selective toxic affinity which uranium possesses for this portion of the tubule. These changes vary from those of granular degeneration with edema as vacuolation, to one of complete epithelial necrosis. As is usual at such a period of an acute uranium intoxication, the dominant change in the proximal convoluted tubule epithelium is one characterized by vacuolation and a commencing epithelial necrosis.







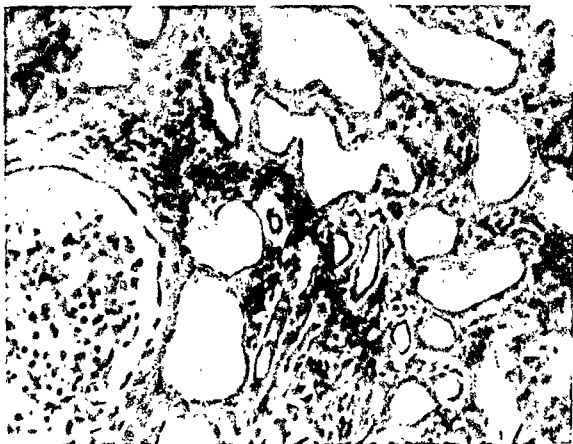


FIG. 3. COLORED MICROPHOTOGRAPH, ZEISS,  $\times 600$

The microphotograph is from the kidney of the animal of experiment 12 after this animal had effected a survival from the acute uranium injury to the kidney as represented by the colored microphotograph (fig. 2). Figure 3 was made after this animal of experiment 12 had received and also effected a survival from 15 mgm. of bichloride of mercury per kilogram given by stomach tube.

In the lower left hand portion of the figure is seen approximately three-fourths of an enlarged glomerulus. The glomerular capillaries show an increase in nuclei and with Mallory's stain the earlier stages in connective tissue formation. Bowman's membrane and the periglomerular area are thickened by the formation of connective tissue. There is both a patchy and diffuse intertubular formation of this tissue. The normal structure of the proximal convoluted tubules of the nephron has undergone as a result of the repair process from the uranium intoxication an entire change in morphology. The segments of these tubules surrounding the glomerulus are no longer lined by cuboidal, specialized cells, but by the atypical, flattened type of cell or by an epithelial tissue either showing partial or no cell differentiation, but remaining as a new lining for these tubules as syncytial structures. The nuclei in such repaired and resistant epithelium to bichloride are usually elongated or definitely ovoid and stain intensely. This figure was made from sections of the kidney of the animal of experiment 12 that was in the fourteenth week of survival from the uranium injury and on the fourteenth day of the acute bichloride of mercury intoxication. The urine of the animal at this time and for ten days prior to this, had shown mercury in an amount sufficient to induce an order of acute necrosis to normal epithelium of the proximal convoluted segment of the nephron as is shown by figure 1 of a normal control animal.







tained from the kidney at the end of 2 weeks, 2 months or 4 months following the commencement of the bichloride intoxication. Such renal tissue studied at the 2-week period has shown the glomerular capillaries fairly uniformly distended with blood without capillary rupture and the gross extravasation of blood into the capsular space. The intracapillary and periglomerular fibrosis resulting from the uranium intoxication persists. In this tissue from animals at the later dates of study, 2 and 4 months, these chronic changes have increased in extent and in occasional glomeruli connective tissue hyalinization has commenced. The striking change of resistance is to be found in the epithelium of the proximal segment of the tubule, that segment for which bichloride in a normal animal has a high degree of selective toxic affinity. The atypical, flattened epithelial cells, or such tissue in the form of syncytial structures, shows no evidence of injury from the bichloride. The cells remain in the flattened, evenly and intensely staining state with deeply staining, usually oblong nuclei that was developed by repair incident to the acute uranium injury. Epithelial cells in this location of the tubules associated with, but not necessarily dependent upon a change in their morphology, have acquired a resistance to the degenerative influence of bichloride of mercury (fig. 3). In such tissue the cells of the loops of Henle and of the distal convolution of the nephron which have not been severely injured as a result of the uranium intoxication and which have therefore not had the degree of damaging influence which leads to an atypical type of cell repair, have effected a repair of a normal cytological order. Such epithelium, as indicated by cloudy swelling, granular changes and rarely vacuolation, give evidence of its inability to withstand the action of bichloride of mercury. Finally, histological studies made at later periods from renal tissue of these animals which have withstood the acute uranium injury by developing an effective type of resistant epithelial repair against the action of bichloride of mercury, have shown no indication of a reversion of the atypical cell type back to a cell type of normal morphology for the proximal convoluted segment of the nephron. The glomeruli and the periglomerular areas as well as the intertubular spaces show a progressive increase in connective tissue formation.

#### DISCUSSION AND SUMMARY

A review of the experimental data presented in this study permit the following conclusions:

1. The normal control experiments have shown that when bichloride of mercury is given to the dog by stomach tube in the amount of 15 mgm. per kilogram, it leads to a dominating and fairly selective injury to the epithelium of the proximal convolution of the nephron which results in a variable but severe degree of necrosis of the epithelium of this segment. The cells of the loops of Henle and of the distal convolution participate to a much less extent in this injury. The glomeruli, in comparison with



the degree of injury to the proximal convolution, are relatively spared. The capillaries in these structures are acutely engorged with blood, dilated, but rarely show endothelial disintegration. This type of action, especially the specific epithelial factor, is constant for normal dogs.

2. The histological study of the kidneys of dogs intoxicated with 4 mgm. of uranium nitrate per kilogram subcutaneously, indicates in such animals a severe renal injury in which also the epithelium of the proximal convolution of the nephron shows the severest degree of participation. A certain number of such animals fail to survive. Those which effect a survival do so in part because the renal injury leads to an epithelial repair process in the severely injured proximal convolution of an atypical and abnormal order for the epithelium in the segment. The new cells are flattened, stain evenly and intensely, and have ovoid deeply staining nuclei. In such areas cell differentiation may not be perfect. A sufficiently severe order of injury by uranium to convoluted tubule cells would appear to prevent such cells from accomplishing a normal type of epithelial repair. A changed or metaplastic type of repair has taken place which resembles embryonic epithelial tissue and suggests a reversion to this order of tissue for purposes of repair in such severely injured areas of the tubule.

The glomeruli, which have shown but slight evidence of an acute uranium injury, do show the result of such an injury through repair by the development of intracapillary and periglomerular changes of a productive order. Such animals have a satisfactory degree of renal function, not only for this purpose as such, but for the maintenance of the life of the organism as a whole. In this instance tissue repair which, when contrasted with the normal, would have to be designated disease, has stabilized the animal in life.

3. These animals which have recovered from an acute uranium injury to the kidney have been given the same amount of bichloride of mercury by stomach tube (15 mgm. per kilogram) that was given to the normal control animals. The mercury content in the blood and in the urine are similar to the values obtained from normal control animals. A certain number of these uranium animals which were given bichloride succumbed in the early days of the intoxication from a severe gastroenteritis and an associated shock. In such animals there was but slight evidence of renal injury and no evidence of the selective epithelial bichloride injury to the atypical epithelium in the proximal convolution. Such animals as survived this initial period, characterized by a gastroenteritis, also effected their permanent survival without the development of the characteristic renal injury from bichloride. Mercury was constantly present in the blood of all of the dogs and present in the urine (with two exceptions) in concentrations similar to those observed in the control animals in which it



served as an effective renal poison. The experiments indicate that the repaired kidney resulting from a severe acute injury from uranium nitrate has the ability to expose itself to, and eliminate in the urine, bichloride of mercury in amounts sufficient to severely injure, as indicated by necrosis, the epithelium of the proximal convolution of the nephron in the normal kidney. In such repaired kidneys in which the repair process has taken place in this segment of the tubule by the formation, not of a normal, but of an atypical type of epithelium, an epithelium changed morphologically and probably chemically as well, an acquired epithelial resistance has been established on the part of the kidney in this portion of the tubule for which bichloride of mercury normally has a highly selective toxic action.

The exact mechanism of the elimination of mercury by the mammalian kidney is not known. Should it be eliminated by a process of glomerular filtration, then we must assume that during its passage through the lumen of the tubule, with or without tubular reabsorption, the altered epithelium of the proximal convolution is resistant to its action. Or, if we assume that the process is one of epithelial secretion at this segment which normally shows evidence of injury by it, then the same assumption must be made. Finally, it is conceivable that the atypical epithelium in this segment is of such a changed order that it fails to subject itself to the mercury action. With such an assumption to explain the fact of acquired epithelial resistance, the inference must be made that the atypical epithelium has so changed from the normal in its chemical constitution that it has lost the specific affinity which it normally has for this poison.

In this study, as well as in others which have been referred to, the acquired resistance of the epithelial tissue has been associated with certain cell metaplasias, changes in cell morphology. Such observations have recently been strengthened by the work of Selye (17) who has demonstrated that a resistance on the part of the mouse kidney to bichloride of mercury may be established by the subcutaneous injection of testosterone prior to the use of a certainly toxic dose of the poison. The characteristic degenerative changes in the epithelium fail to develop. Selye does not attribute this protection to the change in height of the epithelial cells lining the parietal wall of Bowman's capsule, or to the induced renal epithelial hypertrophy, but to a protection to the tubular cells against the damaging action of bichloride. Bunting and Longley (18) have made observations of a rather similar character in connection with the acquired resistance of the epithelium of the rat kidney to arsphenamine. Such an acquired resistance by this tissue is not associated with a change in gross morphology. The studies of Francis and Stuart-Harris (19), (20) on the nasal histology of epidemic influenza virus infection in the ferret, show that, associated with a change in epithelial cell type in this loca-



tion, either due to the action of the virus in high concentration or to the action of a chemical agent in which the columnar, ciliated epithelium is replaced by a flattened type of cell, the animals acquire a resistance to the virus when it is instilled into the nose. The altered cell type is not only resistant to the local action of the virus, but closes this avenue for the entrance of the virus into the organism. These investigators furthermore demonstrated that, when the flattened type of cell which was resistant to the virus reverted back to the normal order of cell for this location, such cells lost their resistance to the local virus effect and the animals became susceptible to the influenza virus infection. A very similar observation to this has come from this laboratory (21). If the livers of dogs be severely injured by the use of uranium nitrate, the repair process results in the formation, not of normal polyhedral cells, but of atypical, flattened cells and syncytial epithelial structures. Such a repair may have a normal functional expression. This type of repair process is entirely resistant to chloroform when such animals are starved for 24 hours and given chloroform for 3 hours by inhalation. Normal animals when subjected to such a procedure invariably show a central necrosis of the liver lobules involving one-half to two-thirds of their area. After a period of several months the flattened and atypical epithelium of repair in areas of the lobules reverts back to the normal polyhedral type of cell. When biopsy material from the liver indicates that such a morphological change has taken place it has been demonstrated that in such areas, of cell reversion there has occurred a loss in the acquired resistance of the epithelium. If such an animal is starved for 24 hours and given chloroform by inhalation for even  $1\frac{1}{2}$  hours there develops a necrosis in those epithelial areas in which the cells have effected a reversion to the normal type.

An analysis of the experiments presented in this paper as well as certain observations which have been discussed as emanating from other laboratories would indicate that change in cell form may or may not be responsible as such for an acquired fixed cell resistance to chemical agents. It is the opinion of the writer that such a concept is not only a workable one, but a true one. An acquired cell resistance associated with a change in morphology should be looked upon as a gross manifestation of such chemical modifications within cells as to express themselves structurally as changed morphology and chemically as an acquired resistance. The life within a cell is not a static, inelastic process, but an everchanging one, attempting to so adapt and relate itself through chemical change that normal life as normal function may express itself. Under the influence of chemical agents of a normal or of an abnormal order for a given organism, and for tissues in a given location, the chemistry of the living substance may be so altered as to favor susceptibility or establish various degrees of acquired fixed cell resistance. It is only when such chemical changes are of such a severe order of modification that they express themselves in terms of transitory or permanent alterations in cell type that atypical



morphology, exerting its influence as changed chemical constitution as well as altered physical form, expresses itself as an acquired tissue resistance.

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# METABOLISM, TOXICITY AND MANNER OF ACTION OF GOLD COMPOUNDS IN THE TREATMENT OF ARTHRITIS

## II. A COMPARATIVE STUDY OF THE DISTRIBUTION AND EXCRETION OF GOLD FOLLOWING THE INTRAMUSCULAR INJECTION OF FIVE DIFFERENT GOLD COMPOUNDS

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Received for publication July 17, 1941

The lack of accurate information regarding the pharmacology of gold has become apparent with the recent increasing use of gold salts in the treatment of arthritis (1, 2). Especially is it desirable and necessary to know more concerning the distribution and excretion of gold when administered by various routes in both man and experimental animals. Previous investigations along this line have been recently summarized by Fleischmann (3). A study of this report indicates that in most instances (4, 6) the amount of gold in various organs was estimated by non-specific chemical or micro-histologic methods. In only one investigation (7) referred to was a reasonably accurate chemical method employed. Further, there has been no satisfactory comparative study of the effects of various gold compounds administered in similar amounts in one species of animal. DeWitt (7) investigated the deposition and excretion of a series of gold cyanide compounds in guinea pigs. However, these compounds are not used clinically and cannot, therefore, be compared to the findings of others (4, 6) who studied the therapeutic preparations of colloidal gold, colloidal gold sulfide, and gold sodium thiosulfate administered in different ways to a variety of animals.

For these reasons it was our purpose to compare the distribution and excretion of gold in the white rat following the administration of five different gold compounds. This was made possible by the use of a recently devised, highly accurate and specific microchemical method for the determination of gold in biological fluids and tissues (8, 9).

### PROCEDURE

Thirty white rats weighing 160 to 170 grams were divided into five equal groups. Each animal was given 14 daily intramuscular injections of 1 mg. of gold; a different gold compound was used for each group. The preparations employed are listed in table 1.

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<sup>1</sup> The Rackham Arthritis Research Unit is supported by the Horace H. Rackham School of Graduate Studies of the University of Michigan.



Each animal was placed in an individual metabolism cage with an arrangement for the quantitative collection and separation of urine and feces. On the day after the last injection the animals were decapitated and the tissues listed in table 2 were rapidly removed, weighed, placed in a Kjeldahl flask and digested with a mixture of nitric and sulfuric acid and later treated with a 30 per cent solution of hydrogen peroxide (Superoxol) until a water clear digest was obtained. The digest was then quantitatively analyzed for gold by the method of Block and Buchanan (8, 9). The urine and feces eliminated during the entire period of study were pooled separately for each animal and were analyzed for gold. Blood secured by decapitation was heparinized and pooled for each group of animals and the gold of the plasma and cells was determined separately.

### RESULTS

The total amount of gold found in the various tissues, urine and feces appears in table 2. Of the 14 mg. of gold administered, our recovery of gold varied from 10.57 to 13.25 mg. If to these values one adds the calculated amount of gold in the plasma (table 4) the recoveries range from 75.5 per cent (gold sodium thiomalate) to 95 per cent (colloidal gold). These high recoveries act as a check on the validity of our procedure and method of analysis. Table 3 shows the amount of gold per gram of the various tissues analyzed.

From these data it is evident that the colloidal preparations behave quite differently from the crystalline compounds. Colloidal gold and colloidal gold sulfide were poorly absorbed, as indicated by the finding of 32.4 per cent of the injected gold at the site of injection in the case of colloidal gold and 40.9 per cent in the case of colloidal gold sulfide (table 2). These values contrast sharply with the comparatively small amounts (1.4 per cent to 7.6 per cent) of the gold of the crystalline compounds which was not absorbed. With all gold preparations, the organs of those analyzed separately containing the greatest amount of gold after absorption were the liver and kidneys, much smaller amounts were found in the spleen and insignificant amounts in the other organs. This is not in agreement with the findings of DeWitt (7) who reported that the greatest amount of gold per gram of tissue occurred in the spleen.

Colloidal gold and colloidal gold sulfide were found to be similar also in that more gold per gram of tissue was found in the liver than in the kidney. In the case of the other three compounds, the greatest quantity of gold occurred in the kidney. In addition, the site of primary pathology following the administration of these substances occurs in the organs where the greatest deposition of gold occurs (10). The grouping of these compounds in regard to deposition is of special interest because it likewise holds true for the relative percentages of urinary to fecal excretion of gold. Thus in the cases of gold sodium thiomalate, gold sodium thiosulfate and sodium succinimido aurate, most of the gold is excreted in the urine, while a lesser amount is excreted in the feces. The reverse holds true in regard to route of excretion in the case of colloidal gold and colloidal gold sulfide. This is of special significance in view of the fact that with substances producing the greatest deposition of gold in the kidney the more important route of excretion is through the urine whereas



TABLE 1  
*Gold compounds studied*

$\text{Na}_2\text{Au}(\text{S}_2\text{O}_4)_2$ Gold Sodium Thiosulfate <sup>1</sup> (37.4 per cent gold)	$\text{Au}_2\text{S}_3$ Colloidal Gold Sulfide <sup>2</sup> (87.0 per cent gold)	Au Colloidal Gold <sup>3</sup> (100 per cent gold)
$\begin{array}{c} \text{COONa} \\   \\ \text{H}-\text{C}-\text{S}-\text{Au} \\   \\ \text{CH}_2 \\   \\ \text{COONa} \end{array}$		$\left[ \begin{array}{c} \text{O} \\    \\ \text{C}-\text{CH}_2 \\   \\ \text{N} \\   \\ \text{C}-\text{CH}_2 \\    \\ \text{O} \end{array} \right]_4^- + \text{Na} \cdot 4\text{H}_2\text{O}$
Gold Sodium Thiomaleate <sup>3</sup> (50 per cent gold)		Sodium Succinimido-Aurate <sup>4</sup> (28.82 per cent gold)

<sup>1</sup> Supplied by G. D. Searle & Co., Chicago, Illinois.

<sup>2</sup> Supplied by Hille Laboratories, Chicago, Illinois.

<sup>3</sup> Obtained from Merck & Co., Rahway, New Jersey.

<sup>4</sup> Obtained from Inventions Inc., Chicago, Illinois.

TABLE 2

*Average gold content of various tissues, urine and feces following the intramuscular injection of various gold compounds*

TISSUE	GOLD SODIUM THIOSULFATE		GOLD SODIUM THIOSULFATE		SODIUM SUCCINIMIDO AURATE		COLLOIDAL GOLD		COLLOIDAL GOLD SULFIDE	
	mg.	per cent*	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent
Heart.....	0.001	(0.01)	0.003	(0.02)	0.001	(0.01)	0.000	(0.00)	0.000	(0.00)
Lung.....	0.009	(0.06)	0.015	(0.11)	0.006	(0.04)	0.000	(0.00)	0.000	(0.00)
Spleen.....	0.039	(0.28)	0.038	(0.27)	0.016	(0.11)	0.021	(0.15)	0.031	(0.22)
Liver.....	0.281	(2.01)	0.217	(1.55)	0.103	(0.74)	1.304	(9.31)	1.107	(7.91)
Kidney.....	0.254	(1.81)	0.758	(5.41)	0.270	(1.93)	0.123	(0.88)	0.045	(0.32)
Site of injection.....	1.068	(7.63)	0.509	(3.64)	0.196	(1.40)	4.537	(32.40)	5.725	(40.89)
Carcass.....	1.876	(13.40)	1.637	(11.69)	0.437	(3.12)	3.762	(26.87)	5.008	(35.77)
Urine.....	4.957	(35.41)	5.694	(40.67)	9.603	(68.59)	0.325	(2.32)	0.162	(1.16)
Feces.....	2.196	(15.69)	1.698	(12.13)	2.621	(18.72)	0.601	(4.29)	1.060	(7.57)
Total excretion†....	7.153	(51.10)	7.392	(52.80)	12.224	(87.31)	0.926	(6.61)	1.222	(8.73)
Total.....	10.681		10.569		13.253		10.673		13.138	

\* Values calculated as per cent of total gold injected.

† Urine plus feces.

with those substances whose deposition is greatest in the liver, the greater amount of gold is eliminated through the feces. The absolute percentages of fecal excretion in the case of the latter substances, colloidal gold and colloidal



gold sulfide, are not greater than those for the three crystalline compounds. This is probably associated with differences in the degree of absorption of the colloidal preparations compared to the other compounds. In addition, it should be noted that the total excretion of the colloidal gold compounds (colloidal gold, 6.61 per cent, colloidal gold sulfide, 8.73 per cent) is much less than the total excretion of the crystalline substances (gold sodium thiomalate, 51.1 per cent, gold sodium thiosulfate, 52.8 per cent, sodium succinimido

TABLE 3  
*Average gold content per gram of tissue*

TISSUE	GOLD SODIUM THIO- MALATE	GOLD SODIUM THIOSUL- FATE	SODIUM SUCCINI- MIDO AUATE	COLLOI- DAL GOLD	COLLOI- DAL GOLD SULFIDE
	mg. per gram	mg. per gram	mg. per gram	mg. per gram	mg. per gram
Heart.....	0.002	0.005	0.001	0.000	0.000
Lung.....	0.008	0.016	0.005	0.000	0.000
Spleen.....	0.041	0.039	0.020	0.015	0.021
Liver.....	0.038	0.028	0.014	0.124	0.114
Kidney.....	0.215	0.433	0.182	0.060	0.021
Site of injection.....	0.124	0.064	0.018	0.511	0.543
Carcass.....	0.012	0.012	0.003	0.020	0.025

TABLE 4  
*Gold content of blood after intramuscular injection of various gold compounds*

COMPOUND	PLASMA	CELLS	*GOLD PER TOTAL PLASMA VOLUME
	mg. per cent	mg. per cent	mg.
Gold sodium thiomalate .....	12.11	0	0.650
Gold sodium thiosulfate .....	13.72	0	0.686
Sodium succinimido aurate.....	2.12	0	0.106
Colloidal gold sulfide.....	2.38	0	0.119
Colloidal gold. ....	0.20	0	0.100

\* Gold for total plasma volume calculated on the basis that blood volume of a rat is about 10 cc. with 50 per cent hematocrit (12).

aurate, 87.3 per cent.) This definitely indicates a difference in the rate of excretion of the two groups of substances.

Thus from the standpoint of absorption, site of deposition, site of primary pathology, route of excretion and the amount excreted, the five compounds studied can be divided into two groups: colloidal gold and colloidal gold sulfide in one, and gold sodium thiomalate, gold sodium thiosulfate and sodium succinimido aurate in the other. This grouping we believe to be dependent on the physical properties primarily and possibly related to chemical structure. Those substances comprising the first group are colloidal insoluble prepara-



ions while those of the second group are crystalline compounds which although varying in absolute solubility can nevertheless be administered in aqueous true solution.

Table 4 shows the results of analyses of blood plasma and cells for gold. In every instance gold in the blood was found to be exclusively in the plasma. Although this finding is contradictory to the observation of DeWitt (7) that all the gold present in blood was in the erythrocytes, it is precisely what we have found in our studies of human blood (11).

#### CONCLUSIONS

1. The absorption of gold compounds after intramuscular administration in rats appears to be related to the physical properties (specifically solubility) of the compounds injected. The colloidal preparations were absorbed poorly compared to the crystalline salts in aqueous solution.

2. Following intramuscular injection of various gold compounds in amounts to contain equivalent quantities of gold, gold was found in the kidneys and liver in large amounts than in any other organ analyzed; considerably less gold was found in the spleen, and insignificant quantities occurred in other organs analyzed. The amount of gold in the kidneys was greater following administration of the crystalline gold salts and was greater than was found in the liver. After injection of colloidal preparations, gold was found in larger amounts in the liver. (There is direct correlation between these results and the pathology observed in the various organs.)

3. Considerable gold was found elsewhere in the carcass; further studies will be needed to more definitely locate this.

4. Gold is excreted both in urine and in feces. The urine contained much more gold than did the feces in the case of those preparations (crystalline) with which the kidney gold content was greater than the liver; the feces were the chief route of excretion in the case of those substances (colloidal) producing liver gold content greater than kidney.

5. Gold was found only in the plasma of blood.

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## THE SELENIUM CONTENT OF "NORMAL" URINE

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Received for publication July 16, 1941

In the control of industrial exposures to selenium, in which appreciable absorption and excretion of selenium has been demonstrated (1), the quantitative determination of selenium in urine and feces (particularly in the former) plays an important rôle. The subject has an additional interest in its bearing on the problem of "trace" elements in biological materials.

There are few references in the literature to the selenium content of human urine. The finding by Gassmann (2, 3) of amounts of selenium ranging from 0.8 to 1.2 mg. per 100 cc. in the urine of presumably normal adults with no special selenium exposure could not be confirmed by Fritsch (4) who found no selenium in specimens collected from individuals in the same region. Much more recent references by Dudley (5, 6) state: "urine of normal subjects, not in any way subjected to selenium, shows no detectable quantities of selenium," and "the excretion of selenium in the urine is conclusive evidence that workers are absorbing selenium. However, more clinical and experimental laboratory work is necessary in order to establish a method of differential diagnosis based on the quantity of selenium in the urine." He reported values of 0 to 6.9  $\gamma$  (micrograms) per 100 cc. in the urines of workers exposed to selenium in the smelting of copper ore. Manville (7), in a review of the "selenium problem" writes, "It is stated that normal urine does not contain selenium and that if urinary selenium is found, it indicates exposure and absorption." In a study of individuals living in a region known to have considerable amounts of selenium in the soil, Smith *et al.* (8) found urinary selenium values varying from 10 to 200  $\gamma$  per 100 cc.

In the course of our control studies of individuals exposed to an organic selenium compound (9), it was found that following an appreciable absorption and excretion of selenium (the latter occasionally as high as 800-900  $\gamma$  per 100 cc. of urine) the amount of selenium in the urine decreased after a few days to weeks to "trace" levels. In the earlier stages of this study no selenium was found by the methods then employed in the urines of individuals with no industrial selenium exposure. Later, the finding of small amounts of selenium in the urines of individuals several months after exposure had ceased stimulated a re-examination of "normal" urine for possible selenium content.



## METHOD

The "dead-stop end point" electro-titrimetric modification by Wernimont and Hopkinson (6) of the iodine-thiosulfate method described by Mathews, Curl and Osborn (11) was used: "The organic matter in a 100 cc. sample of urine was destroyed with concentrated sulfuric acid in the presence of mercury. The selenium was separated from the digestion mixture by distillation in the presence of hydrobromic acid and then precipitated (using sulfur dioxide and hydroxylamine hydrochloride), filtered, washed, and redissolved in a solution of hydrobromic acid and bromine. The bromine was destroyed and an excess of standard sodium thiosulfate solution was added along with a little potassium iodide. The unused sodium thiosulfate was titrated with standard potassium iodate, using the dead-stop method for determining the end point (6)." Amounts of selenium as low as 1 to 2  $\gamma$  can be estimated from the characteristic green to yellow to red turbidity in the precipitation step if viewed in a Tyndall beam, a check on the final determination. In addition, a rough estimation of the selenium content can be made from the density of the pink film in the porcelain filter crucible after filtration (12).

## RESULTS

In a preliminary survey, 78 random specimens of urine collected for routine medical urinalysis from male industrial employees in an area having no known seleniferous soil<sup>1</sup> and no other known selenium exposure were analyzed for selenium content. In about half of these, the estimations were made turbidimetrically, and the remainder were determined electrometrically. Of these specimens, 70 per cent contained selenium. Six specimens (8 per cent) contained more than 10  $\gamma$  per 100 cc. In another experiment, the selenium from fourteen 100 cc. aliquots of pooled urine was precipitated, collected, and titrated, giving a total value of 40 micrograms or an average of 2.8  $\gamma$  per 100 cc. sample.

Four consecutive first morning specimens were obtained from each of ten healthy male employees with no industrial selenium exposure. Samples were collected in ground glass stoppered pyrex flasks which had previously been scrupulously cleaned and finally rinsed with a bromine-hydrobromic acid solution. Every specimen contained selenium.

Consecutive specimens of urine for approximately a twenty-four hour period were obtained from three other healthy male adults. Selenium was found in all of the fractional specimens but one, a two-hour sample of relatively large volume and low specific gravity.

The distribution of urinary selenium concentrations in the specimens obtained from these two groups is shown in table 1. The mean values 5.0 and 4.2  $\gamma$  per 100 cc. are in good agreement. The slightly higher values shown for the first group of samples may be explained by the fact that these samples represent longer average collection periods, all of the specimens being first morning samples (i.e., the urine collected in the bladder overnight). Nearly 50 per cent of the samples in the first group contain 5 or more micrograms per cent of selenium, with 40 per cent of the samples in the second group at this level.

<sup>1</sup> Rochester, New York.



Some question may be raised as to the validity of the small amounts of selenium demonstrated, since the lower values found were at the limit of sensitivity of the method. As a check on the tritrations, the selenium found in the individual samples was pooled and the total amount of selenium determined. This value, 271  $\gamma$ , represented 90 per cent of the summed individual values.

When the concentration of selenium is plotted against the rate of urinary output the result is interesting (graph 1). There is a definite inverse relationship, the greater the rate of urinary output, the less the selenium concentration. This relation was noted in our studies involving much greater urinary

TABLE 1  
*Distribution of normal urinary selenium (concentration)*

4 CONSECUTIVE 1ST MORNING SAMPLES, 10 MALE ADULTS		CONSECUTIVE SPECIMENS FOR 24-HOUR PERIOD, 3 MALE ADULTS	
$\gamma$ /100 cc.	Distribution	$\gamma$ /100 cc.	Distribution
0 - 0.9	2	0 - 0.9	1
1.0- 1.9	4	1.0-1.9	1
2.0- 2.9	3	2.0-2.9	2
3.0- 3.9	6	3.0-3.9	2
4.0- 4.9	7	4.0-4.9	3
5.0- 5.9	4	5.0-5.9	3
6.0- 6.9	9	6.0-6.9	3
7.0- 7.9	1		
8.0- 8.9	2		
11.0-11.9	1		
15.0-15.9	1		
Total.....	40	Total.....	15
Mean.....	5.0	Mean.....	4.2
Standard deviation .	$\pm 2.9$	Standard deviation	$\pm 1.8$

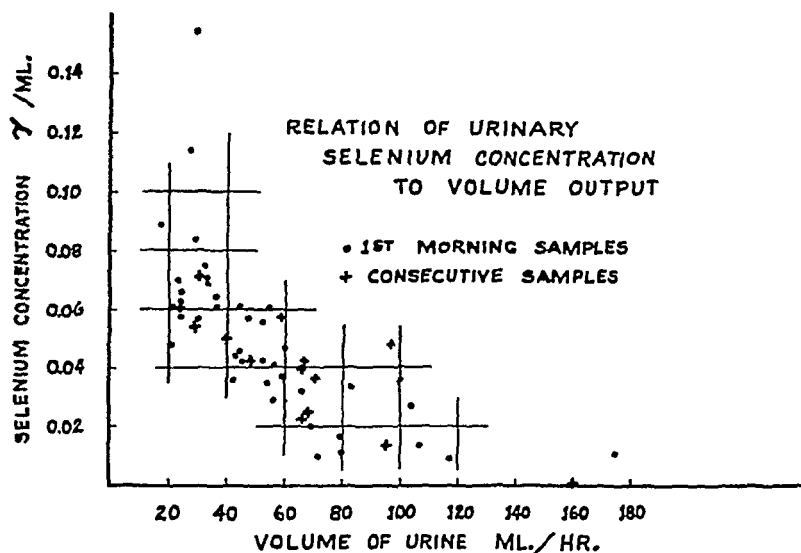
excretion of selenium, and the relative constancy of this excretion rate permitted a good estimate of the total 24 hour excretion from the analysis of two or three smaller samples, of which the total volume and duration of collection was known.

The data suggests that selenium is handled by the kidney as a "non-threshold" substance, similar to creatinine and sulfates, since the amounts excreted in a given time are remarkably constant and independent of the concentration of urine.

The distribution of the excretion rates of selenium for the two groups of specimens is shown in table 2. Values are in micrograms per hour with means of 2.1 and 2.3 respectively. It will be observed that these values arrange themselves in a much closer approximation to a "normal curve" distribution than do the "concentration" results.



The variability in the concentration and excretion rate of urinary selenium in consecutive samples throughout the day is shown in table 3. It is seen that



GRAPH 1

TABLE 2

*Distribution of normal urinary selenium (excretion rate)*

4 CONSECUTIVE 1ST MORNING SAMPLES, 10 MALE ADULTS		CONSECUTIVE SPECIMENS FOR 24-HOUR PERIOD, 3 MALE ADULTS	
$\gamma$ /hour	Distribution	$\gamma$ /hour	Distribution
0 -0.4		0 -0.4	1
0.5-0.9	2	0.5-0.9	
1.0-1.4	6	1.0-1.4	1
1.5-1.9	11	1.5-1.9	4
2.0-2.4	12	2.0-2.4	3
2.5-2.9	5	2.5-2.9	4
3.0-3.4	2	3.0-3.4	1
3.5-3.9	1	3.5-3.9	
4.0-4.4	1	4.0-4.4	
		4.5-4.9	1
Total.....	40	Total.....	15
Mean.....	2.1	Mean.....	2.3
Standard deviation.....	$\pm 0.8$	Standard deviation.....	$\pm 1.0$

the rate of excretion of selenium is in general more constant than the concentration or the total amount of selenium per sample.



Robinson (13) showed that samples of wheat from different parts of the world all contained selenium,—varying from 0.1 to 1.9 parts per million. Wheat products were suspected as one source of the above selenium excretion

TABLE 3

*Normal urinary selenium output; consecutive specimens for 24-hour period, 3 male adults*

	HOURS COLLECTED	TOTAL VOLUME	$\gamma$ Se PER 100 CC.	$\gamma$ Se PER TOTAL VOLUME	$\gamma$ Se PER HOUR
		cc.			
J. R. P.	7:35 a.m.-11:30 a.m.	125	6.9	8.7	2.2
	4:15 p.m.	210	6.0	12.6	2.7
	6:55 p.m.	185	3.7	6.9	2.6
	10:30 p.m.	340	4.8	16.5	4.7
	6:30 a.m.	470	5.8	27.3	3.4
Total. ....	23 hours			72	3.1 (av.)
J. H. S.	7:15 a.m.-12 Noon	320	4.2	13.3	2.8
	5:00 p.m.	235	4.2	9.8	2.0
	11:15 p.m.	410	3.9	16.1	2.6
	7:30 a.m.	200	6.2	12.5	1.5
	1:40 p.m.	415	2.3	9.6	1.5
Total. ....	30½ hours			61.3	2.0 (av.)
F. L. O.	7:05 a.m.-1 p.m.	175	5.5	9.7	1.6
	5:00 p.m.	160	5.1	8.1	2.0
	7:00 p.m.	320	0.0	0.0	0.0
	12:15 a.m.	500	1.4	7.0	1.3
	7:40 a.m.	510	2.5	13.0	1.7
Total. . . .	24½ hours			37.8	1.5 (av.)

TABLE 4

*Selenium analyses in bread and flour*

	$\gamma$ Se PER 100 GRAMS
White bread, brand A. ....	27.6
White bread, brand B. ....	28.2
Cracked wheat bread . . . . .	36.8
Rye bread (dark) . . . . .	39.2
Bleached flour, brand X . . . . .	26.2
Bleached flour, brand Y. . . . .	31.8
Bleached flour, brand Z . . . . .	32.8

by normal "non-exposed" individuals. An extensive study of the dietary sources of selenium is beyond the scope of this paper, but a few samples of wheat products purchased in Rochester, N. Y., were analyzed for selenium.



The results are shown in table 4, ranging from 26 to 39  $\gamma$  per 100 grams of dry product, or from 0.26 to 0.39 parts per million.

All of the above samples were obtained from normal healthy male adults, none of whom were taking any medication immediately preceding or during the period of these studies. Physical examination, complete hematological studies, and routine medical urinalyses were made on all the subjects and were essentially negative. There is no evidence that the absorption and excretion of amounts of selenium of the order found in this study results in any symptoms or other harmful effects.

#### SUMMARY

1. Selenium was found in a high percentage of the urine specimens obtained from normal, healthy male adults who had no industrial or other obvious selenium exposure. This finding is in contrast to recent references on the subject.

2. The concentrations of selenium averaged from 4 to 5  $\gamma$  per 100 cc. of urine, with a range of 0 to 15  $\gamma$ .

3. The rate of urinary selenium excretion is relatively constant and is quite independent of the urinary volume, suggesting that selenium is treated by the kidney as a "non-threshold" substance similar to sulfates and creatinine. The mean rate was 2.2  $\gamma$  per hour with relatively slight variability.

4. A dietary source of selenium was found in wheat products, with selenium values ranging from 26 to 39  $\gamma$  per 100 grams dry weight, or 0.26 to 0.39 parts per million.

5. No harmful effects were observed which could be correlated with the absorption and excretion of such amounts of selenium as were demonstrated in this study.

Since the completion of this study we received a personal communication from H. W. Lakin of the Division of Soil Chemistry and Physics, U. S. Dept. of Agriculture, stating that they had observed quantities of selenium ranging from 0.2 to 7.0  $\gamma$  per 100 cc. of urine obtained from individuals with no known exposure to selenium. These data are included in U. S. Dept. Agr. Tech. Bull. 758 entitled, "Selenium Occurrence in Certain Soils in the United States, with a Discussion of Related Topics: Fifth Report" which is to be published soon.

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- (13) ROBINSON: Ind. & Eng. Chem., 28: 736, 1936.



amount of the previous dose remains. This interval usually varies from 2 to 4 weeks. In those cases in which the rate did not return to the control level, the interval of 4 weeks or longer was taken arbitrarily. In cases with regular sinus rhythm, an interval of 4 to 7 weeks elapsed between courses. In all cases with sinus rhythm, the T-wave returned to the control form before the next course was given.

It is unlikely that the reaction of the heart to a dose of digitalis is significantly altered by previous doses when such intervals elapse. Nevertheless the order of administration of different specimens was varied so as to avoid error in one direction that might result should this assumption prove to require revision.

Three types of effects were used to measure the degree of action: (1) slowing of the ventricular rate in patients with auricular fibrillation; (2) changes in the T-wave of the electrocardiogram in patients with regular sinus rhythm; (3) toxic effects (vomiting). We have shown in previous studies that these are valid means of measuring that property of digitalis which accounts for the improvement of heart failure (2, 3, 4).

*Cases with auricular fibrillation.* Only those patients were selected who had a rapid ventricular rate when they were not under the influence of digitalis. They were put to bed in the hospital. The routine examinations were made including blood, urine, X-ray and electrocardiograms. The patients were weighed at intervals and the water exchange was observed by measurement of the daily intake and output. The ventricular rate was counted at the apex for a full minute with the stethoscope 3 or 4 times a day, in each case after a period of quiet. This was done for a period of 7 days or longer until a fairly constant level was reached or it was clear that the ventricular rate was mounting. A single oral dose of digitalis was then given at one time in the morning, after which similar counts at the apex were made at intervals of about an hour throughout the day. In the subsequent days the counts were made 3 or 4 times a day in a manner similar to that of the control period. This was continued until the ventricular rate again reached the levels of the control. As stated above, the duration of the period of elimination varied for different patients from about 2 to 4 weeks. At this point the next course of digitalis was given in the same way and a similar record of rate changes was made.

In patients with heart failure the usual improvement took place during the action of digitalis. In the period of elimination the heart rate mounted, and with that the signs of failure reappeared. The degree of heart failure for a given ventricular rate prior to the initial digitalization was often more marked than prior to the subsequent ones, although in some instances the recurrence of the signs and symptoms of failure followed the mounting heart rate quite closely.

In a previous study (5) we showed that slowing of the ventricular rate is a sensitive index of the degree of digitalis action only at the higher rate levels. When the rate is reduced to about 70 or 80 a minute, a moderate increase in the dose of digitalis often fails to produce further slowing. An attempt was therefore made in these experiments to keep the dosage small enough to secure effects in the more sensitive range, although, not knowing in advance the tolerance of any given patient, we were often unsuccessful, and were compelled to repeat the experiment with different doses. In some cases we increased the dose in order to enable us to distinguish the potency of two specimens by the occurrence of minor toxic effects.

Where toxic symptoms did not occur, we considered the level to which the rate declined as the indication of the degree of digitalization, although there is the possibility that reduction in the number of beats per minute may also be an important factor. In one instance the total duration of the curve of rate changes following the dose of digitalis was used as a means of comparison.

There were 8 patients with auricular fibrillation. They received from 2 to 5 courses of digitalis in the manner described above, in all 29 such courses. The groups of comparisons required periods of hospitalization which varied from about 1 to 5 months in the different patients.



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# THE RELATIVE ACTIVITY OF DIGITALIS PREPARATIONS IN THE FROG, THE CAT, AND MAN, AND ITS BEARING ON THE PROBLEM OF BIO-ASSAY AND SO-CALLED DETERIORATION

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Received for publication August 1, 1941

The practical object of the official method for the standardization of digitalis is to insure such uniform potency of the drug that equal amounts of different specimens may be expected to produce substantially similar effects in any given individual. In a preliminary publication (1) we pointed out that the use of frogs for the assay of digitalis leaf or the tincture, as is the case in the official method of the United States Pharmacopeia XI, is not satisfactory. We stated that a relationship between the potency of a specimen of digitalis and the standard preparation (U.S.P. XI Reference Powder) which exists when both are tested on the frog is not necessarily the same as that found when they are tested on man. On the other hand, the results obtained with the cat method in the case of digitalis leaf or tincture apply to humans much more satisfactorily.

The evidence for these views is presented in the present report.

## METHODS

*Subjects.* These were selected from a total case load of approximately 1500 cardiac patients in active attendance at our cardiac clinics. Table 1 summarizes the characteristics of the 16 patients that were used. In several additional patients the study had to be abandoned because of intercurrent infections and other complications unrelated to the digitalis. All but one had organic heart disease. Some had auricular fibrillation, some a normal sinus rhythm. Some were without signs of heart failure; others had moderate to advanced grades of heart failure with congestion.

*Method.* The general plan was to make comparisons of different specimens on one and the same patient. The patient was digitalized, an interval was then allowed to elapse sufficient for the elimination of the drug, and the patient was then redigitalized with the next preparation.

There are no precise means of determining the period for complete elimination of digitalis. In auricular fibrillation the return of the rate to the control level is the most satisfactory measure. Our experience justifies the belief that the return of the ventricular rate to the control level in an uncomplicated experiment (uncomplicated by such factors as infection, hyperthyroidism, exercise), indicates that no significant



TABLE 1  
Summary of cases used in the study

NUM- BER	NAME	AGE  years	SEX	WEIGHT  pounds	DIAGNOSIS*	HEART FAILURE WITH CONGES- TION
1	F. R.	58	F	157	Arteriosclerosis, enlarged heart, auricular fibrillation	Moderate
2	R. B.	28	F	118	Rheumatic, mitral stenosis and insufficiency, aortic insufficiency, enlarged heart, auricular fibrillation	Advanced
3	A. A.	28	F	163	Rheumatic, mitral stenosis and insufficiency, tricuspid insufficiency, enlarged heart, auricular fibrillation	Advanced
4	N. C.	37	M	138	Hypertension, mitral insufficiency, enlarged heart, auricular fibrillation	Advanced
5	G. N.	52	F	152	Rheumatic, mitral stenosis and insufficiency, enlarged heart, auricular fibrillation	Moderate
6	V. R.	69	M	168	Arteriosclerosis, coronary sclerosis, enlarged heart, auricular fibrillation	Moderate
7	S. G.	47	F	182	Rheumatic, mitral stenosis and insufficiency, enlarged heart, auricular fibrillation	Moderate
8	A. P.	19	M	130	Rheumatic, mitral stenosis and insufficiency, enlarged heart, auricular fibrillation	None
9	A. Y.	16	M	150	Rheumatic; heart disease not established, regular sinus rhythm	None
10	G. S.	35	F	153	Hypertension, enlarged heart, regular sinus rhythm	None
11	I. S.	53	F	165	Hypertension and arteriosclerosis, dilated aorta, enlarged heart, regular sinus rhythm	None
12	B. G.	56	F	134	Hypertension and arteriosclerosis, enlarged heart, regular sinus rhythm	None
13	A. S.	36	M	142	Arteriosclerosis, coronary thrombosis, regular sinus rhythm	None
14	I. K.	21	M	148	Congenital, regular sinus rhythm	None
15	S. S.	64	M	174	Arteriosclerosis, coronary sclerosis, regular sinus rhythm	None
16	I. R.	25	F	132	Rheumatic, mitral stenosis and insufficiency, enlarged heart, regular sinus rhythm	None

\* The diagnoses are based on "Nomenclature and Criteria for Diagnosis of Diseases of the Heart," 1939.



amount of the previous dose remains. This interval usually varies from 2 to 4 weeks. In those cases in which the rate did not return to the control level, the interval of 4 weeks or longer was taken arbitrarily. In cases with regular sinus rhythm, an interval of 4 to 7 weeks elapsed between courses. In all cases with sinus rhythm, the T-wave returned to the control form before the next course was given.

It is unlikely that the reaction of the heart to a dose of digitalis is significantly altered by previous doses when such intervals elapse. Nevertheless the order of administration of different specimens was varied so as to avoid error in one direction that might result should this assumption prove to require revision.

Three types of effects were used to measure the degree of action: (1) slowing of the ventricular rate in patients with auricular fibrillation; (2) changes in the T-wave of the electrocardiogram in patients with regular sinus rhythm; (3) toxic effects (vomiting). We have shown in previous studies that these are valid means of measuring that property of digitalis which accounts for the improvement of heart failure (2, 3, 4).

*Cases with auricular fibrillation.* Only those patients were selected who had a rapid ventricular rate when they were not under the influence of digitalis. They were put to bed in the hospital. The routine examinations were made including blood, urine, X-ray and electrocardiograms. The patients were weighed at intervals and the water exchange was observed by measurement of the daily intake and output. The ventricular rate was counted at the apex for a full minute with the stethoscope 3 or 4 times a day, in each case after a period of quiet. This was done for a period of 7 days or longer until a fairly constant level was reached or it was clear that the ventricular rate was mounting. A single oral dose of digitalis was then given at one time in the morning, after which similar counts at the apex were made at intervals of about an hour throughout the day. In the subsequent days the counts were made 3 or 4 times a day in a manner similar to that of the control period. This was continued until the ventricular rate again reached the levels of the control. As stated above, the duration of the period of elimination varied for different patients from about 2 to 4 weeks. At this point the next course of digitalis was given in the same way and a similar record of rate changes was made.

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In a previous study (5) we showed that slowing of the ventricular rate is a sensitive index of the degree of digitalis action only at the higher rate levels. When the rate is reduced to about 70 or 80 a minute, a moderate increase in the dose of digitalis often fails to produce further slowing. An attempt was therefore made in these experiments to keep the dosage small enough to secure effects in the more sensitive range, although, not knowing in advance the tolerance of any given patient, we were often unsuccessful, and were compelled to repeat the experiment with different doses. In some cases we increased the dose in order to enable us to distinguish the potency of two specimens by the occurrence of minor toxic effects.

Where toxic symptoms did not occur, we considered the *level* to which the rate declined as the indication of the degree of digitalization, although there is the possibility that *reduction* in the number of beats per minute may also be an important factor. In one instance the total duration of the curve of rate changes following the dose of digitalis was used as a means of comparison.

There were 8 patients with auricular fibrillation. They received from 2 to 5 courses of digitalis in the manner described above, in all 29 such courses. The groups of comparisons required periods of hospitalization which varied from about 1 to 5 months in the different patients.



*Cases with regular sinus rhythm.* Eight patients were selected for this purpose whose electrocardiograms showed fairly high T-waves in leads 1 and 2. They were ambulant. After a control tracing they received a daily dose of digitalis leaf in a capsule for 7 days, following which another electrocardiogram was made. During the succeeding 4 to 7 weeks they were without digitalis, in order to insure complete elimination. The same type of experiment was then repeated with another specimen of digitalis. The effects upon the RT interval and the T-wave produced by the two specimens were then compared, using lead 1 or 2, depending upon which showed the greater effects. The inspection of the tracings was made by the "blind test," the comparative changes being recorded without knowledge of the drug which applied to any particular tracing.

TABLE 2  
*Specimens of digitalis used in this study*

SPECIMEN	CAT UNIT POTENCY*	PERCENTAGE OF U.S.P. POTENCY BY THE OFFI- CIAL FROG METHOD	AMOUNTS OF "REFERENCE POW- DER" AND TEST SPECIMEN OF DIGITALIS LEAF THAT ARE EQUIVALENT IN POTENCY BY	
			Official frog method	Cat method
Reference powder U.S.P. XI . . .	55 mgm.	135†		
N. Y. Heart Association Leaf #7.	85 mgm.	52†	1:2.6	1:1.5
Tincture digitalis Y . . . . .	0.67 cc.	50†	1:2.7	1:1.2
Tincture digitalis M . . . . .	0.63 cc.	63†	1:2.13	1:1.14

\* The cat method used in all of these assays was the one in use in this laboratory and is carried out as follows: Six cats are used for each specimen. The cats are in apparent good health. They are not pregnant. Their weights range between 2 and 3.5 kgm. They are neither emaciated nor obese. They have not been fed for 24 hours. They are lightly anesthetized with ether, so lightly that their pupils react to light and the corneal reflex is present, the voluntary musculature is not relaxed, and the animal occasionally moves the tail or shows some other voluntary movement. The drug is injected intravenously at such a rate that death with ventricular fibrillation, attended by a convulsion, results in a period of from 60 to 90 minutes. To accomplish this the drug is so diluted with physiological salt solution that the total estimated volume is 15 cc. per kilogram and an amount equal to  $\frac{1}{15}$  of the estimated fatal dose (1 cc. per kilogram of cat) is injected every 5 minutes until death. The injections are made from a graduated burette through a cannula inserted into a vein of the hind leg. Each injection is made rapidly (in a few seconds).

† This figure is obtained by means of the factor stated on the label of the official ampoule of U.S.P. "Reference Powder."

‡ We are indebted to Dr L. C. Miller, of the Food and Drug Administration, for these assays.

*Drugs.* Table 2 shows the characteristics of the four specimens of digitalis used in the study. The Reference Powder of the United States Pharmacopeia XI was chosen because of its high potency by the frog and the cat method. The New York Heart Association specimen of digitalis is the one in use among the member clinics of the association which care for about 20,000 cardiac patients. It differs from the Reference Powder in that it has fairly high potency by the cat method but very low potency by the U.S.P. frog method. The tinctures of digitalis Y and M were included as specimens of commerce showing striking discrepancy between the results by the frog and the cat method; tincture Y was about 82 per cent of the potency of the Reference Powder by the cat method, but only 37 per cent by the frog method. Such preparations were chosen



since it was the object of the study to ascertain whether preparations showing different potencies by the frog method would disclose such differences in man.

### RESULTS

Decision as to the best way of summarizing the results of this study presented something of a problem. The details of the results in different patients were not all the same, and diversified patterns of response required description. Also, there was the fact that in some individuals it was found possible to secure evidence which was complete in itself, while in others a sufficient number of repetitions of courses of digitalis could not be made so that the evidence could not stand alone. It did not seem, therefore, that illustrative cases would

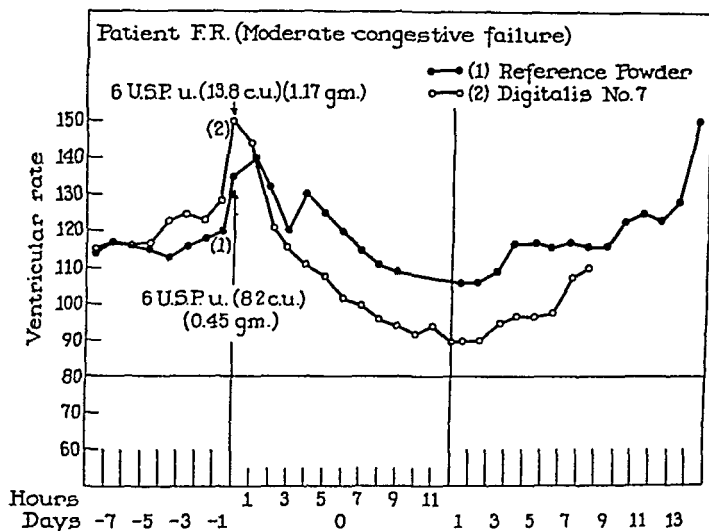


FIG. 1

convey adequately the force of the evidence on which our conclusions are based. We are therefore presenting the results of all the experiments without selection, in the form of 11 condensed charts.

The numbers on the curves indicate the sequence of digitalizations in each patient with auricular fibrillation. Missing numbers represent digitalizations carried out in connection with another study. The digitalis was given in a single dose at the point on the first vertical line.

*Figure 1.* One of the two curves shows a greater effect than the other. They are both produced by the same number (six) of U.S.P. units of two specimens of digitalis. However, the difference in the two curves corresponds to the dosage in terms of cat units. The greater effect was caused by 13.8 cat units and the lesser effect by 8.2 cat units.



Figure 2. In this patient there were 4 courses of digitalis with 2 specimens. In the upper pair of curves the doses of the 2 specimens in terms of U.S.P. units were different, one being 76 per cent greater than the other (5 and 8.8 U.S.P. units respectively), but the

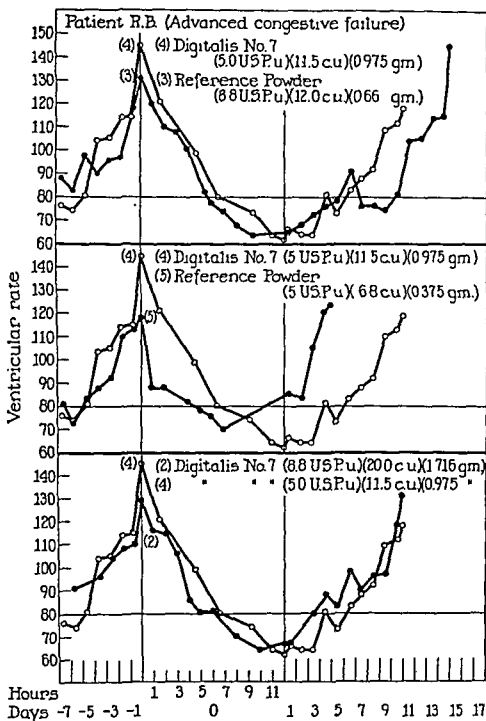


FIG. 2

results were identical and corresponded to the doses in terms of cat units which were also practically identical (11.5 and 12 cat units respectively).

In the middle pair of curves the doses of the 2 specimens in terms of U.S.P. units were kept the same (5 U.S.P. units), but the effects were different, and again corresponded



to the doses in terms of cat units which were also different (6.8 cat units for the lesser effect in curve number 5 and 11.5 cat units for the greater effect in curve number 4).

The lower pair of curves compares the effect of a dose of digitalis which lowers the ventricular rate to normal levels, with that of a 76 per cent larger dose of the same specimen. With respect to the changes in rate, the effects are indistinguishable. While this patient was able to distinguish, by changes in heart rate, a dose of 6.8 from 11.5 cat units of digitalis (middle pair of curves), he was unable to distinguish 11.5 from 20 cat units by this means. The failure to distinguish the different doses in this pair of curves is due to the fact which we have already referred to, that in the lower range of heart rates the sensitiveness of this method of comparison is greatly reduced.

*Figure 3.* In this patient, there were 3 courses of digitalis arranged in 2 pairs of curves. In the upper pair, one curve (number 4) shows a greater effect than the other (number 6). The greater effect represents 8.8 U.S.P. units and the smaller effect 6

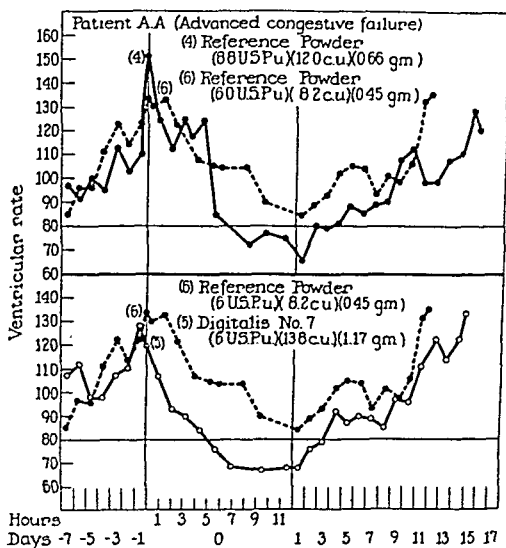


Fig. 3

U.S.P. units of the same specimen, namely, Reference Powder. The heart rate changes clearly reveal a 47 per cent difference in dose in this patient.

In the lower pair, one curve (number 5) also shows a greater effect than the other (number 6), although they represent the same number of U.S.P. units (6 U.S.P. units), but this time, of two different specimens of digitalis. The difference in effects again corresponds to the cat unit potency of the two specimens, the greater effect being produced by 13.8 cat units and the lesser effect by 8.2 cat units, a difference in dosage of 63 per cent in terms of cat units.

*Figure 4.* In this patient there were four courses of digitalis arranged in three sets of curves. Three specimens were used. The unconnected points on the chart represent paroxysms of regular sinus rhythm. Of the upper pair of curves, one (number 4) shows a somewhat greater effect than the other (number 1), although they represent the same number of U.S.P. units (7 U.S.P. units). The greater effect is caused by the larger number of cat units (16 cat units as against 9.5 cat units). The difference is not marked



because even the smaller dose was sufficient to reduce the rate to the relatively insensitive range.

The middle three curves represent three specimens of digitalis, each given in the same number of cat units (9.5 cat units). The effects are practically identical, even though, in U.S.P. units, the doses show wide divergence, from 3.2 to 7 U.S.P. units, a difference of 119 per cent.

This patient was able to distinguish a much smaller actual difference in potency, as shown in the lower pair of curves in which two different doses of the same specimen were

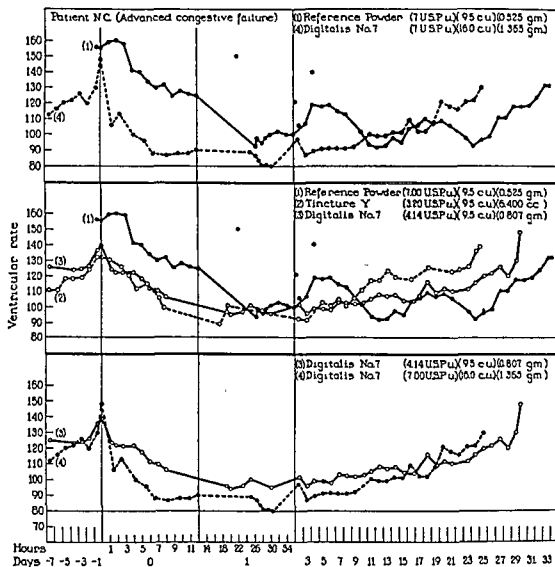


FIG. 4

compared. The one representing the 69 per cent larger dose (number 4) shows a small but distinctly greater effect, the more significant since all the effects in this patient were in the relatively insensitive range.

Since one of these three preparations was a tincture and the others the dried leaf, a comparison was made of the leaf and tincture of the same specimen in the same patient. Figure 5 shows that the effects of the tincture and of the leaf are indistinguishable.

Figure 6. In this patient two specimens of digitalis were compared, the Reference Powder, and a tincture of digitalis which was very weak by the frog method, namely tincture Y. A similar number of cat units of each was given (11.9 cat units). At the



end of 48 hours the effect was only moderate and a similar dose of each was repeated (at the point on the third vertical line). The additional dose produced a much greater effect, indicating that by means of heart rate changes, this subject was able to reveal the effect of a 100 per cent greater dose. This gives some indication of the sensitiveness of the method in this patient. The curves for the two preparations, however, are practically identical, even though, in terms of U.S.P. units, each dose of Reference Powder was 118 per cent larger than each dose of tincture Y.

Figure 7. In this patient two specimens of digitalis were compared in four courses. These are arranged in two sets of curves. The upper two curves are almost indistinguishable. Each represents the effect of 8.8 U.S.P. units of digitalis, but the cat units

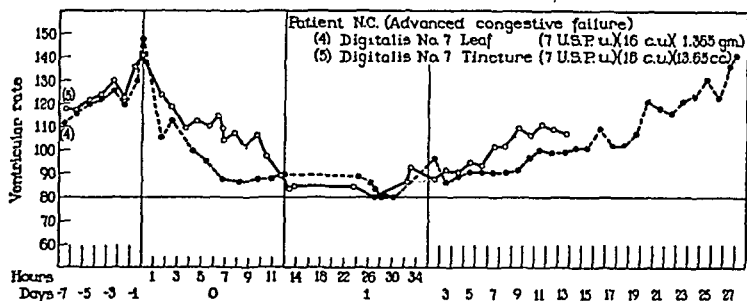


FIG. 5

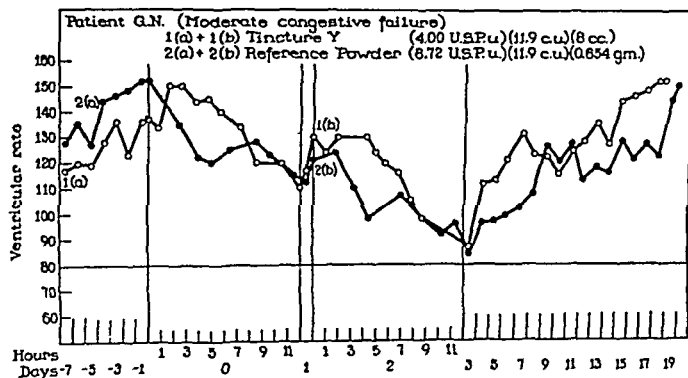


FIG. 6

in one case are 12 (curve number 1) and in the other, 20 (curve number 2). This was the first experiment which suggested that the results of the frog method might apply to man, while those of the cat method might not. This experiment proved deceptive.

Since the rate was reduced to about 70 a minute in each case, an insensitive level in which even a large difference in dosage of the same preparation may not be revealed by heart rate changes, it was desirable to repeat this experiment in a more sensitive region of the dose-effect curve. But the rate criterion was not promising in this patient because the control rates were not very high. The experiment was then repeated with doses of both drugs 50 per cent larger, as shown in the lower pair of curves. The rate changes were not very different, but now the preparation, (curve number 3) representing the



larger number of cat units caused toxic effects (vomiting), whereas the one representing the smaller number of cat units did not. A similar number of U.S.P. units of two preparations produced different effects. This case, therefore, is not an exception as it first appeared.

**Figure 8.** In this patient there were four courses of digitalis with two specimens of the drug. The results are arranged in three pairs of curves. The middle pair shows the effect of 8.8 U.S.P. units of Reference Powder in two courses. The curves are practically identical.

In the upper pair of curves, the same number of U.S.P. units (8.8) of two specimens were given. The curves are different. In one (curve number 1) satisfactory digitalization was produced, whereas in the other (curve number 2) the rate declined to lower levels and vomiting resulted. The lower pair of curves shows a repetition of these two

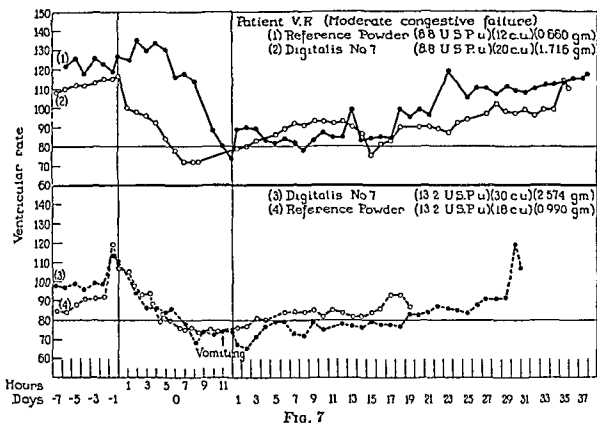


FIG. 7

digitalizations. The results are practically identical with the first two. In each case the preparation representing the higher number of cat units produced the greater effect (lower rate and vomiting), although the same number of U.S.P. units were represented in the amounts of digitalis that were given.

**Figure 9.** In this patient there were three courses of digitalis with three specimens. Two curves (numbers 2 and 3) are similar, showing satisfactory digitalization, with a decline of the rate to the level of about 80 a minute. They are different from curve number 1, which shows a much greater effect, namely, decline of the rate to below 60 a minute and repeated vomiting over a period of 48 hours. Curves 1 and 2 which show such different effects represent the same number of U.S.P. units (10 U.S.P. units) of two preparations, and curves number 2 and 3 which show similar effects represent dissimilar doses in U.S.P. units, namely 10 and 4.56 U.S.P. units respectively. Again, the intensity of effects corresponds to the dosage in cat units.



*Figure 10.* This represents a case which tests more directly the formulations arrived at in the previous experiments. The patient received two courses of Reference Powder, 8.8 U.S.P. units in each. The two curves (numbers 1 and 3) are practically identical. These were used for comparison with a commercial tincture of digitalis (tincture M) found by the Food and Drug Administration to be of substandard potency, namely, 63 per cent U.S.P. potency by the frog method. Two types of comparisons were made. The first was based on the assumption that substandard strength by the U.S.P. frog method does not necessarily apply to man. Accordingly a dose of 8.8 cc. was given.

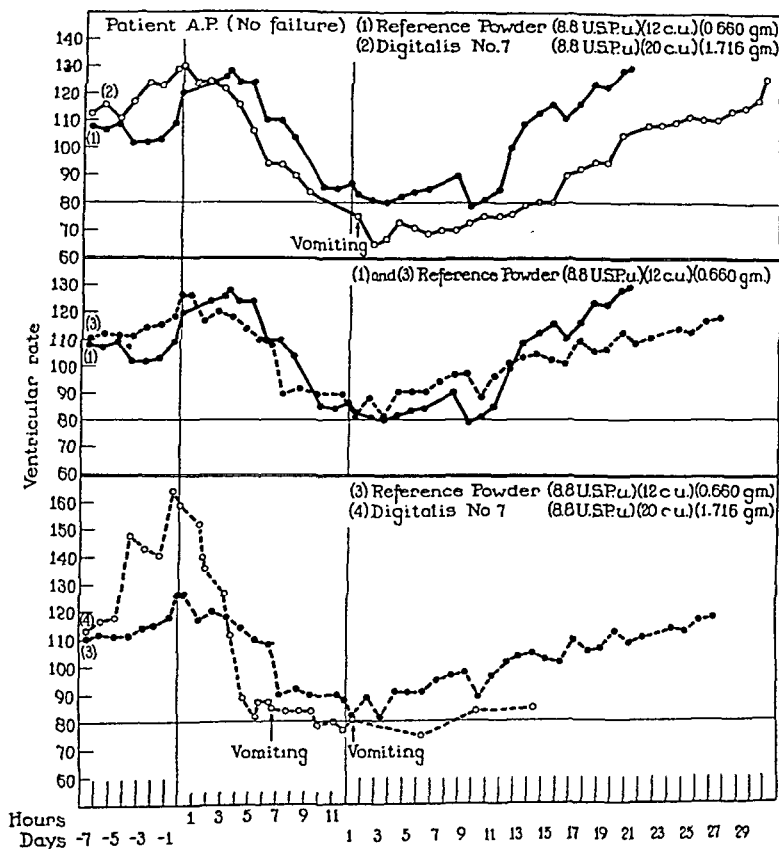


FIG. 8

The similarity of curve number 9 with the previous curves shows that this dose produced in man an effect of 8.8 U.S.P. units, although it contains only 63 per cent or 5.5 U.S.P. units. The second comparison was based on the assumption that a substandard potency by the U.S.P. frog method is substandard in man. Accordingly the dose was correspondingly increased and 14 cc. was given. Although this represents only 8.8 U.S.P. units of tincture M, the effects were greater (lower rate and vomiting) (curve number 10) than with a similar number of U.S.P. units of the Reference Powder. In short, when a tincture found to be of substandard potency by the frog method was



assumed to be of substandard potency in man, and the dose correspondingly increased, the patient was poisoned. Again, the greater effect was caused by the larger number of cat units.

**Figure 11.** This shows the results obtained with 8 patients in whom the New York Heart Association digitalis #7 and the Reference Powder were compared by the method previously described for patients with regular sinus rhythm. The 8 patients are

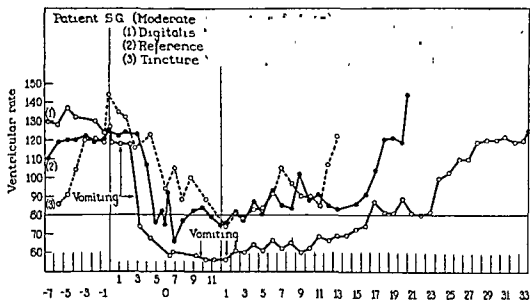
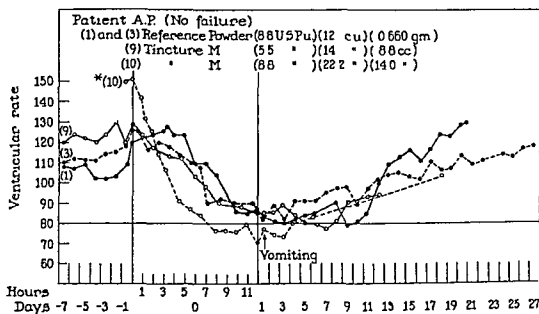


FIG. 9



\* Without digitalis 21 days. Walking about for 1 week prior to dose.

FIG. 10

(read from above downward): Yo (lead 1), Sp (lead 1), Sta (lead 1), Go (lead 2), Ste (lead 2), Kl (lead 2), Stei (lead 1), Ru (lead 2). The order of administration of the 2 drugs was varied, the following receiving the Reference Powder first: Yo, Sp, Kl and Ru. Column 1 represents the control tracing in each case; column 2, after one week with a daily dose of 2 U.S.P. units of Reference Powder. Column 3 represents another control tracing after a period of from 4 to 7 weeks allowed for the elimination of the first drug; column 4, the effect of 2 U.S.P. units of digitalis #7 given in the same way. It may be



noted that 5 of the 8 patients (first 5) are clearly able to distinguish these two specimens by changes in the T-wave. The digitalis #7 produced the greater effects on the T-wave. These results, therefore, confirm the observations made on the patients with auricular fibrillation, and show that the U.S.P. unit (frog method) does not apply, that a U.S.P.

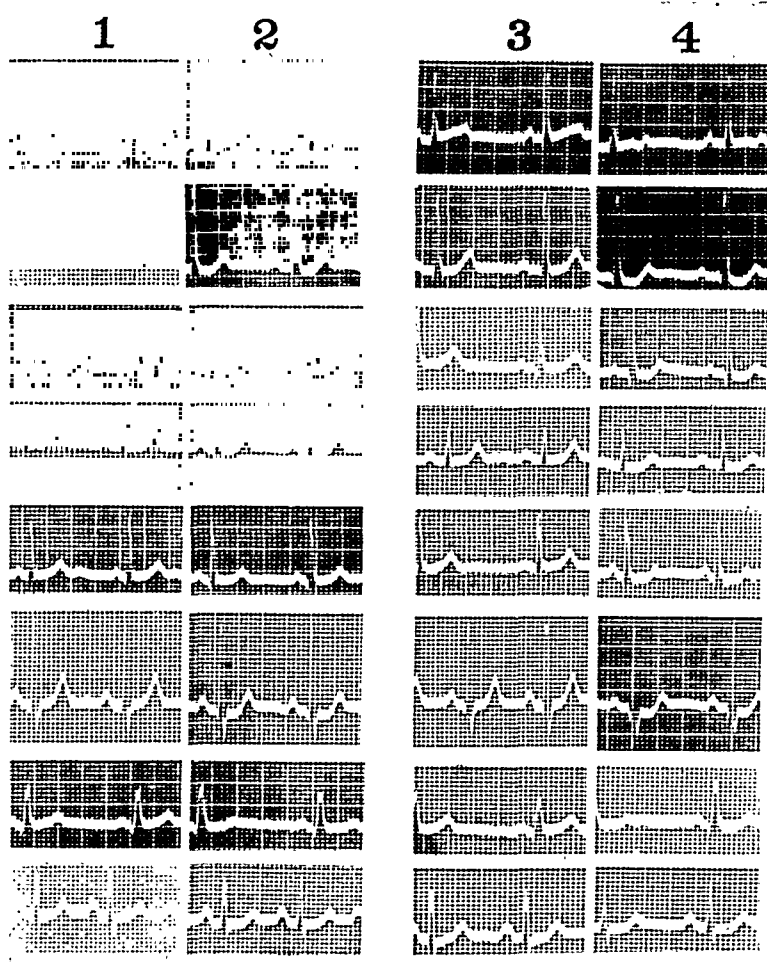


FIG. 11

unit of one specimen of digitalis may produce greater effects than a U.S.P. unit of another in man. It may also be noted that in terms of cat units the greater effect was produced by the larger number of cat units, since the 2 U.S.P. units of Reference Powder represented only 2.7 cat units, whereas the 2 U.S.P. units of digitalis #7 represented 4.5 cat units.



## DISCUSSION

It has been generally taken for granted that the results of the U.S.P. frog method of assay of digitalis are applicable to humans although no systematic investigation to test this assumption had ever been made. Eggleston (6) maintained that the results of the cat method conform more strictly to those in man, and it has long been known that the frog and cat methods do not always yield similar results. Our attention was again focused upon this matter by some observations which we made about two years ago when we assayed, by the cat method, a number of outstanding tinctures of digitalis now in use in the United States, following the technique employed in this laboratory (table 3). Their potency was quite variable; the strongest was about three times as potent as the weakest. They were all labeled U.S.P. XI tincture of digitalis, presumably, therefore, of the same potency by the frog method, within the limits of tolerance allowed by the Pharmacopeia.

TABLE 3  
*Potency of tinctures of digitalis (U.S.P. XI)*

SPECIMEN	CAT UNIT POTENCY; TINCTURE
	cc.
1 John Wyeth.....	0.35
2a Parke Davis.....	0.36
2b Parke Davis .....	0.46
3 Squibb .....	0.58
4a Digitol (Mulford).....	0.58
4b Digitol (Mulford).....	0.86
5 Lilly.....	0.60
6 "Tr. Digitalis U.S.P. XI".....	0.96

A serious question was presented by this finding. Digitalis assayed by the cat method and prescribed in cat units is widely employed throughout the United States. The mass experience of the New York Heart Association is of interest in this connection. As already stated, these clinics care for about 20,000 cardiac patients yearly, and during the past 14 years they have used more than 17 million digitalis tablets standardized by the cat method. The general experience with these tablets is that they run clinically uniform from one batch to another. In view of the fact that this experience is based on several specimens of digitalis which varied in potency but were assayed by the cat method and were so prepared that each tablet contained the same number of cat units, it is strongly suggestive that the results by the cat method are applicable to humans. If this is so, it follows that the results by the frog method may not apply, since the results by the two methods are frequently different.

In order to resolve this question, it was necessary to apply the results of the



two animal assay methods in experiments on human subjects, so devised as to insure the detection of differences that may exist between specimens. We should stress the need for suitable experiments on man since in the routine clinical use of digitalis, fairly marked differences in potency of preparations escape detection for reasons stated in an earlier paper (7). A distinctive feature of the present experiments is the fact that the source of error arising from the varying sensitiveness of different patients does not enter, since, while a group of patients were used, the comparisons were made upon one and the same patient. These experiments show that when the frog and the cat method give different values for the potency of a specimen of digitalis, the figure obtained by the cat method applies to humans, while that obtained by the frog method does not. There were no exceptions. Three specimens of digitalis found to be very weak by the frog method were of full strength when administered to humans. In all cases, the intensity of digitalis action corresponded to the number of cat units and not to the U.S.P. units (frog method).

In some experiments in which specimens were found weak by the frog method but strong by the cat method, the low potency by the frog method was disregarded; the drug was given in doses as though it were of full strength by the frog method. It produced effects in man consistent with full potency. When, however, it was assumed that low potency by the frog method signified a low potency in man and the dose accordingly increased, the patient was poisoned.

This matter bears upon some practical problems. It is encountered in connection with the law enforcement of digitalis assay standards. A specimen of digitalis in commerce found to be substandard by the frog method often proves to be of full strength by the cat method. The results in this study show that such specimens are also of full strength in man (tinctures Y and M). One way to avoid this violation would be to increase the potency of such a preparation to full U.S.P. strength (for frogs) as a result of which the material becomes over-strength for humans. The problem of the manufacturer is clearly presented in a recent communication from Thompson (8):

"Since pharmaceutical manufacturers are required by law to distribute Tincture Digitalis in such a manner that it will comply with the U.S.P. requirements during a reasonable shelf life of the product (six months or more), it is obviously necessary to manufacture the product at an original potency of almost 200 per cent of the U.S.P. requirement. Since the product cannot leave the manufacturer at twice U.S.P. potency for obvious reasons, it must be aged to allow the initial rapid deterioration to take place. When the potency level has reached reasonable stability at approximately the U.S.P. potency requirement (approximately 60 per cent of the original potency of the batch), it is subjected to its final minor adjustment of potency and distributed in commercial channels. This procedure is obviously essential to the manufacturers in order to avoid citations by the Food and Drug Administration, and indeed, such practice has been verbally advocated by official authorities as the answer to the deterioration problem. . . . the practice is a dangerous one . . . ."



May not the foregoing facts explain, in part, the extraordinary potency and variability of commercial U.S.P. tinctures of digitalis when assayed by the cat method, as shown in table 3?

The results of the present study also have a bearing on the problem of so-called deterioration of digitalis. It is well known that with aging, some specimens of the tincture of digitalis—and it is probable that this also applies to leaf—grow weaker as tested by the frog method (9, 10). The manufacturer often discards it, resulting in great waste of digitalis. There is evidence that this material which has grown weaker by the frog method retains its potency by the cat method, and also in humans (11, 12).

In the previous communication (1) we indicated that the final statement regarding the relative potency of digitalis materials may have to be obtained from comparisons on humans directly. Such comparisons must be made in the case of purified glucosides, since their absorption from the gastrointestinal tract shows extremely wide variations; in the case of some glucosides, nearly complete absorption; in the case of others, the absorption of only a small fraction of a dose. The present study shows that in the comparison of different specimens of digitalis leaf and the tincture, the cat method yields results which are in closer conformity with the results in humans than the frog method. Of the animal assay methods, the cat method appears, from existing evidence, to be the most satisfactory guide to the therapeutic potency of digitalis leaf and the tincture. However, there are theoretical objections to the cat method as well, and there is evidence that the cat method may at times prove misleading. The method involves intravenous injection, and a preparation with a comparatively large amount of unabsorbable active material may show high potency by the cat method but low potency in humans when given by oral administration (13, 14). Such preparations appear to be rare. We have never encountered them.

#### SUMMARY AND CONCLUSIONS

1. A method is described by which the potency of digitalis preparations can be compared in humans using one and the same subject for the comparisons, and thereby eliminating error arising from individual differences in susceptibility.
2. This study shows that the frog method for the assay of digitalis gives results which are not applicable to humans. One preparation may be half as strong as another by the frog method, but the two may have equal potency in man.
3. This study also shows that the cat method for the assay of digitalis leaf and the tincture gives results which more nearly parallel their potency in man.



*Acknowledgments.* The authors wish to acknowledge their indebtedness to Dr. A. A. Epstein, Dr. I. W. Held, and Dr. J. J. Hertz, chiefs of the medical services, for their generous coöperation, and to the several members of the visiting staffs and house staffs for their liberal contribution of time and effort.

These studies were supported in part by the Digitalis Fund of Cornell University Medical College, to which contributions have been made by Sharp and Dohme, John Wyeth and Brother, Sandoz Chemical Works, the Warner Institute for Therapeutic Research, Laboratoire Nativelle, Lederle Laboratories, Inc., and the Ciba Pharmaceutical Products, Incorporated.

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# THE RESPIRATORY EFFECTS OF MORPHINE, CODEINE, AND RELATED SUBSTANCES

## VIII. THE EFFECT OF SUBSTITUTIONS ON CARBON-14<sup>1</sup>

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Received for publication April 19, 1941

In the molecule of morphine two of the most important radicals, pharmacologically, are the secondary alcoholic group at carbon-6 and the phenolic hydroxyl group at carbon-3 (1). If the alcoholic hydroxyl is replaced or muzzled a dose of the resulting compound is equivalent to nearly ten times as much morphine for depressing the respiration of rabbits; if the phenolic hydroxyl is muzzled the product is equivalent to about one-tenth as much morphine. The nature of the group used to muzzle or replace the hydroxyl matters very little. It is reasonable to ask whether or not a hydroxyl group attached at another point in the molecule is as significant as these, especially since Eucodal, a derivative of morphine carrying one at carbon-14, has come into clinical use. Carbon-14 is a member of the nitrogen-containing ring. Three of its valences are connected with other nuclear carbon atoms, while its fourth, in morphine, bears a hydrogen atom, which by indirect methods is replaceable. We have studied the respiratory effects, in rabbits, of 9 derivatives of morphine in which acetoxyl, hydroxyl, or bromine was substituted for this hydrogen atom; we found that hydroxyl here had nothing like so large or so consistent an influence upon potency for depressing respiration as in the other two positions. The results are a contribution toward a growing mass of knowledge, that still awaits interpretation, about chemical structure in relation to pharmacological activity among compounds derived from morphine.

### THE DRUGS

The simplest plan for pursuing the question which we have proposed is to compare the effects of changing the radical at carbon-14, while the rest of the molecule remains con-

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<sup>1</sup> The work reported in this paper is part of a unified effort by a number of groups to solve the problem of drug addiction. The participating organizations have been the Rockefeller Foundation, the National Research Council, the United States Public Health Service, the United States Bureau of Narcotics, the Massachusetts Department of Health, the University of Virginia, and the University of Michigan.



TABLE I  
*Physical properties of the drugs*

DRUG	MOLECULAR WEIGHT	BASIC FRACTION	MELTING POINT	APPROXIMATE SOLUBILITY AT 25°C. IN PARTS PER 100 OF H <sub>2</sub> O	OPTICAL ROTATION		
					[α] <sub>D</sub>	Temperature	c.
321. Acetylhydroxycodine hydrochloride	391.63	per cent 90.8	°C. 260-261* with decomp.	8	+15.7	25	0.87 H <sub>2</sub> O
391.† Diacetyldihydrohydroxycodine-B acid tartrate	569.29	70.5	160-165	10	-78	29	0.72 H <sub>2</sub> O
392.† Diacetyldihydrohydroxycodine-C acid tartrate	569.29	70.5	187-188	2	-67	29	0.80 H <sub>2</sub> O
320. Hydroxycodine hydrochloride	367.63	85.2	272-276*	8	-90	24	0.86 H <sub>2</sub> O
322. Dihydrohydroxycodine hydrochloride (= Eucodal)	351.62	89.6	270-272 with decomp.	very soluble	-123	29	0.67 H <sub>2</sub> O
323.† Dihydrohydroxycodine-A	317.18	100	301-302*		-64	29	0.42 10% acetic acid
324.† Dihydrohydroxycodine-B	317.18	100	143		-136	26	0.60 10% acetic acid
370.† Dihydrohydroxycodine-C	317.18	100	163-164		-152	23	0.56 ethanol
31. Bromocodine	376.16	100	155-157	insoluble			

\* *In vacuo.*

† New compounds first prepared in Dr. Small's laboratory.



stant; but then only four drugs, one corresponding to each radical, would contribute to the answer. To increase the scope of the study we have included drugs of six molecular types, all belonging to the codeine family of morphine derivatives: codeinone, dihydrocodeinone, dihydrocodeine, dihydroisocodeine, acetyldihydrocodeine, and acetyl-

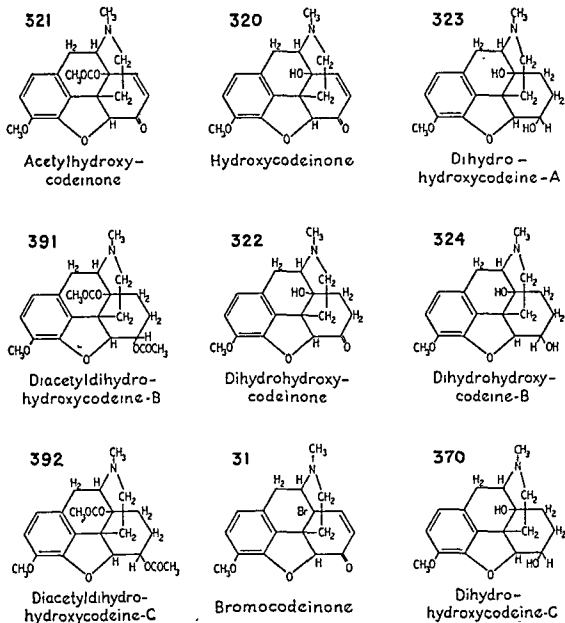


FIG. 1. STRUCTURAL FORMULAS OF THE DRUGS

The method of preparation of 323 must have this derivatives is not clear. of dihydrohydroxycodeine hydroxycodeine directly or crosswise.

dihydroisocodeine. Theoretically the possible combinations of 4 substituents, acetoxyl, hydroxyl, bromine, and hydrogen, with 6 different molecular types, make 24 compounds. Actually, only 13 of these were available to us. The respiratory effects of 5, dihydrocodeinone (#154), dihydrocodeine (#20), dihydroisocodeine (#51), acetyldihydrocodeine (#367), and acetyldihydroisocodeine (#390), are already described elsewhere



(2, 3, 4, 5). The effects of the other 8 will be described here for the first time; and, in addition, those of dihydrohydroxycodine-A, because the method of its preparation relates it closely to dihydrohydroxycodine-B and -C, though its structure is so uncertain that it must be put in a class by itself. The eight and this supernumerary one are listed in table 1. For a discussion of their chemistry see the paper by Lutz and Small (6). Their probable structural formulas are shown in figure 1, drawn all as bases, though some were received in the form of salts, as table 1 shows. Dr. Small, in whose laboratory they were prepared, furnished the information on their physical properties which is summarized in table 1. Each drug is designated not only by name, but also by a number arbitrarily assigned, for making rapid reference to tables and graphs.

### THE EXPERIMENTS

The experiments were performed as described in the first paper of this series (7). To recapitulate, unanesthetized rabbits, tied supine on cushioned boards, wore masks containing flap valves through which they could be connected either with a tank of room air or a tank containing 6-10 per cent  $\text{CO}_2$  and 20-40 per cent  $\text{O}_2$ . When they rebreathed the room air, soda lime removed expired  $\text{CO}_2$ , but the soda lime container was short-circuited when they breathed the mixture high in  $\text{CO}_2$ . From either tank their respiration was recorded on a kymograph by a Krogh spirometer. The procedure in each experiment was first to record respiration for 6 minutes while the rabbit breathed room air and then for another 6 minutes while it breathed the artificial mixture. After about 3 minutes of the latter the rabbit's respiration had usually become steady at a nearly constant rate and depth. Then the animal was disconnected from the tanks and injected subcutaneously with a dose of drug. One hour later we again recorded its respiration while breathing air and  $\text{CO}_2$  mixture. The pre- and post-drug records yielded measurements from which the oxygen consumption of the animals while they breathed room air, and their respiratory rate, tidal volume, and minute volume during their connection with both tanks, were calculated. Incidentally, heart rates were counted. Any of these functions after injection, expressed as a percentage of the same function before injection, we speak of as a drug effect.

For each drug, the effect of varying dose was studied from a level at which the depression was nil or very small, up to a level at which danger of killing the rabbits, or a tendency of the drug to cause convulsions, or lack of drug, limited further extension of the range. The amounts injected formed a rough geometrical progression, spaced usually according to multiples of the pattern, 1, 2, 5, 10, 20, .... (With all expressions of dosage in this paper, let *per kgm.* be understood.) Usually, eight rabbits were used to determine the effects of each dosage. No animal was injected more than once within a week. We observed precautions already described (7) to secure accurate, reproducible, and homogeneous data.

In this series of papers dealing with the respiratory effects of derivatives of morphine there has not hitherto been a discussion of the ways in which the circumstances of the experiments, the weight, sex, diet, and posture of the rabbit, and the temperature of the room, affect the results. It might be thought, since every feature of respiratory behavior after the drug is considered only in its relation to the normal respiration, for the same animal, under the same set of conditions, that the influence of such factors, exerted alike upon pre- and post-drug observations, would largely cancel one another. This is not true for rate of respiration, at least, because all these factors play some part in determining the rate at which the normal rabbit breathes, and that, in turn, plays a part in determining the degree to which a drug depresses the rate. The higher the initial rate, the greater is the depression, in relative as well as absolute units. Meissner (8) and others have reported this. Data obtained in our own laboratory with morphine



hydrochloride illustrate it. Fifteen rabbits, which had been breathing room air with frequencies between 29 and 79 per minute, received 0.05 mgm. of morphine. Their respiratory frequencies an hour later, expressed as percentages of the normal, were plotted against the normal. They showed a definite tendency to fall about 12 units for every increase of 10 in the initial rates. After doses of 0.5 and 5.0 mgm., given to 12 and 8 individuals respectively, the depression changed about 8 units for every increase of 10 in initial rate. At 50 mgm. no perceptible correlation existed. The experiments are too few to permit exact prediction of the relation of post-drug rate to pre-drug rate at a given dose, but they indicate that the faster a rabbit breathes normally the greater will be the depression following an injection of morphine or related compounds. This fact has two consequences: the *variability* of the data is increased by it, and the mean *level* of the results is partly determined by such experimental conditions as affect initial rate.

First we shall discuss the difficulty with respect to *variability*. The temperature of the laboratory varied between 21° and 26° on different days, though the change was negligible on any one day. In the experiments now being reported, and in later ones, about 20 per cent of the rabbit population was male, the normal respiratory rate of males being somewhat higher, on the average, than that of females. The weights of the rabbits varied from 2.8 to 3.8 kgm., a spread wide enough to allow detection of a positive correlation of respiratory rate with weight. These factors of sex, temperature, and weight, by acting directly upon normal rates, undoubtedly worked indirectly to make the effects of drugs upon rate more variable than they would have been otherwise. Even if one secured sufficient data with morphine to determine completely the influence of this third dimension, initial rate, upon the dosage-effect relationship, a correction factor obtained for morphine could not be applied to another drug, and the labor that would be necessary to obtain it anew for each drug seems uncalled for. We have dealt with the difficulty by discarding animals with a normal rate higher than 80, and estimating from both control and drug experiments the net variability caused by this along with unrecognized factors. In the general table of results, the standard deviation is given for each average effect.

In our experiments, the effects of morphine upon minute volume were not, at any level of dosage, correlated with the normal minute volume, as effects upon rate were related to the normal rate. It is noteworthy in the table that the data upon minute volume always vary less, as measured by the standard deviation, than corresponding data upon rate.

The correlation of degree of effect with initial rate, besides affecting the *variability* of results, permits the arbitrary selection of experimental conditions partly to determine the mean *level* of the results. For instance, our rabbits breathed about 50 per cent faster on a diet of Purina chow than they did on the ration of oats and alfalfa which was the standard, and 50 per cent faster when held right side up in a sort of pillory than when lying upon their backs in the posture characteristic of the regular experiments. In these two instances, therefore, our choice of conditions probably resulted in a somewhat smaller effect upon rate, for any given dose, than might have appeared with the alternative set of circumstances. The arbitrary bias consequent upon choice of conditions, however, is reproducible.

The validity of comparing the results here reported with those for the older drugs in the series might be questioned. From one to six years intervened between observations, the present authors are different individuals, minor changes in technic have been made. The results of control experiments, exactly like those made with drugs except that only water was injected, were published (7) when these studies were begun. The control results given in table 2 of the present paper were performed at least four years afterward, under the new auspices. Their means and standard deviations agree satisfactorily with the older ones.



## RESULTS

We present the results of the experiments in three different ways: *A.* A complete tabulation (table 2) of all the data that were obtained; *B.* Graphs of two functions, respiratory rate (figure 2) and respiratory minute volume (figure 3), of rabbits breathing room air; *C.* Equations (table 3) which describe selected portions of table 2. Each way has its special disadvantage and its special usefulness. Detailed comparison of the drugs and discussion will be based almost entirely upon the third.

*A.* Table 2 is a complete summary of averages of the effects obtained with the drugs. As in earlier papers of this series, the dosages appear (column 1) in milligrams per kilogram of body weight, to simplify practical application of the work. For theoretical reasons, they have been translated into millimoles also; and, for convenience in plotting, into the logarithm of the number of millimoles (column 2). With each mean effect is the standard deviation, or  $\sigma$ , calculated as  $\pm \sqrt{\frac{S(x^2)}{n-1}}$ , on Fisher's authority (9); and  $n$  (column 3), the number of individual effects contributing. So exhaustive an accumulation of data is of little use in comparing drugs. Scanning the mass, one gathers readily that none of the compounds affected the consumption of oxygen appreciably, even in large doses; and that two of them, bromocodeinone and hydroxycodinone (#31 and #320), caused no respiratory depression of any kind at any dose; but little else appears. In order to gain any clear impression of the relations among the drugs, one must abstract these data in some way. The advantage of table 2 is that it supplies a full statistical description of the observations, provided one assumes, as we think one may, that individual effects were normally distributed about their mean. Hence the reader can, if he prefers, abstract by another method than those we have chosen (*B* and *C*).

*B.* The respiratory rate and the respiratory minute volume of rabbits breathing air changed considerably with variation in dosage, and were therefore chosen for graphic comparison of the compounds. In figure 2, effects upon rate, and in figure 3, effects upon minute volume, are plotted against the logarithm of the number of millimoles of drug injected. The special advantage of the graphs is that they tell at a glance the relation of effect to dosage as found for each drug. With a number of morphine derivatives previously studied, either rate or minute volume plotted against the logarithm of the dose makes an inverted S. The transition from no effect to depression, as dosage increases, seems to be gradual rather than of threshold type, making the head of the S. (This type is well illustrated here (fig. 2) by the rate results of diacetyldihydrohydroxycodine-C (#392), and less strikingly by dihydrohydroxycodinone (#322).) Then, as the dose increases further, the respiration falls rapidly, tracing on the graph a nearly straight line, the body of the S. Finally, if dosage is pushed high enough, increasing restless-



ness in the animals partially offsets depression, so that the resultant respiration begins to return towards normal, putting a tail on the S. With most of the compounds in the present group, except #392 and #322, the onset of effect with increasing quantities appears to be quite abrupt. Then, with still larger amounts, the respiration drops swiftly. The range of doses was not carried far enough in these experiments to discover a return toward normal with any of them.

A few elementary comparisons are possible in the graphs. It is again obvious that bromocodeinone (#31) and hydroxycodeinone (#320), in the amounts tried, lacked all depressant influence. Dihydrohydroxycodeine-A (#323), given in relatively large quantities, lowered rate very slightly, minute volume not at all. The remaining compounds reduced profoundly both rate and minute volume. The difference between the weakest and the strongest among them is about one logarithmic unit, equivalent on an arithmetical scale to a tenfold change. Noticeable is the fact that the weakest, dihydrohydroxycodeine-C (#370), differs chemically from the strongest, dihydrohydroxycodeinone or Eucodal (#322), in the presence of a secondary alcoholic hydroxyl group at carbon-6 in place of the carbonyl group. The contrast exemplifies the usual influence, already alluded to, of that radical in that position.

The disadvantage of the graphs is that they leave the problem of comparing drugs in definite quantitative terms still unsolved. The confusion that results when large numbers of compounds are studied in a single graph is prohibitive.

C. In the two preceding papers of this series (4, 10), the authors fitted straight lines by the method of least squares to such data as fell on the steep, descending, body of the S in the graphs. Results with doses so small as to cause no significant depression, and with doses large enough to complicate depression by activity, were discarded before the calculation. With the depressant members of the present group of drugs we have done likewise, for the functions, respiratory rate and minute volume, on both room air and CO<sub>2</sub>. The values in table 2 upon which calculations have been based are indicated by asterisks. The resulting equations are given in table 3, in two forms: one calculated for dosages in milligrams as in the earlier papers, the other for dosages in millimoles to correspond to figures 2 and 3 here. The last column of the table contains the standard deviation of each line, a value that expresses the "spread" of individual observations, by stating that 68 per cent of those used in calculating fell within that vertical distance from the line.

The constants of these simple linear equations provide a means for describing the effects of different drugs in a succinct and semiquantitative way. The *slope*, *m*, is the rate at which, once depressant dosages are reached, further increase in dose changes the degree of depression. The *intercept*, *b*, is larger in proportion as more drug is required to produce a given effect.



TABLE 2  
Mean effect of the drugs at different dosages

DOSAGE		NUMBER OF ANIMALS	HEART RATE		OXYGEN CONSUMPTION		RATE				MINUTE VOLUME			
Mgm.	Log (mmols. $\times 10^3$ )		Per cent	$\sigma$	Per cent	$\sigma$	Air		CO <sub>2</sub>		Air		CO <sub>2</sub>	
Controls														
0.0		39	100.9	7.8	102.1	9.5	101.5	21.1	101.9	9.3	99.1	14.5	101.7	10.9
Drug #321. Acetylhydroxycodeinone hydrochloride														
0.3	1.885	10	100.6	7.8	105.9	10.2	93.6*	7.8	93.3n*	8.5	96.6*	6.3	95.8n*	11.4
0.5	2.107	10	97.5	7.4	98.9n	13.7	81.4n*	9.5	86.2*	7.9	90.7n*	7.1	85.2*	10.4
1.0	2.408	10	88.1	7.4	100.7	7.8	67.0*	11.4	77.7*	5.9	81.4*	8.7	73.4*	8.4
3.0	2.885	9	82.7	25.3	97.9	9.4	50.7*	15.5	49.3*	19.0	65.2*	12.9	42.9*	16.9
5.0	3.107	11	66.8	14.1	82.8	21.6	25.0*	10.6	31.6*	18.0	43.7*	13.4	27.8*	13.2
a: n = 9														
Drug #391. Diacetylhydroxycodeine-B acid tartrate														
0.3	1.722	8	97.5	5.9	103.9	8.7	98.3*	18.0	99.0*	9.4	95.6*	7.9	96.8*	9.3
0.5	1.944	8	96.0	4.7	95.0	11.1	85.4*	5.6	91.4*	6.7	91.1*	4.2	88.1*	5.7
1.0	2.245	12	99.4	7.5	102.8	10.8	90.8*	9.8	90.8*	10.4	94.2*	9.8	89.5*	10.7
3.0	2.722	8	95.5	9.9	100.6	13.5	66.5*	15.4	75.4*	10.9	80.1*	14.4	76.9*	16.5
5.0	2.944	8	95.6	9.5	95.8	9.6	53.6*	9.8	70.9*	8.9	68.0*	11.1	66.8*	12.5
10.0	3.245	7	82.6	11.1	86.3	7.8	46.7*	9.8	51.3*	11.4	60.9*	10.0	46.1*	16.1
20.0	3.546	7	78.0	11.9	92.6	8.7	36.1*	6.9	44.0*	11.1	55.7*	5.5	41.6*	10.8



Drug #392. Diacetyldihydrohydrocodeine-C acid tartrate

0.05	0.944	8	100.1	3.6	101.1	11.9	96.0	12.8	99.5	11.8	100.6	13.8	102.3	14.6
0.1	1.245	12	98.2	5.2	101.7	11.6	96.5*	17.8	96.7*	8.9	105.3*	21.3	99.5*	8.7
0.3	1.722	8	97.8	5.0	104.5	7.2	90.8*	14.3	97.0*	9.8	96.0*	9.6	93.3*	12.7
0.5	1.944	8	95.4	8.4	99.6	8.4	80.3*	20.9	88.6*	7.5	88.1*	13.7	83.6*	7.8
1.0	2.245	7	95.1	5.0	106.1	18.8	81.0*	20.4	88.0*	8.5	89.9*	10.5	87.4*	9.3
3.0	2.722	8	82.0	8.1	95.5	10.8	47.1*	5.3	59.4*	8.6	69.9*	2.0	55.3*	7.6
4.0	2.847	3	84.3	5.9	99.7	12.8	42.0*	13.8	61.7*	12.4	71.7*	8.6	63.0*	10.2
5.0	2.914	9	80.6	8.0	92.9	16.6	40.2*	9.4	50.6*	10.2	64.6*	9.0	47.7*	8.1
10.0	3.245	8	64.6	18.5	76.0	15.9	24.4*	15.5	23.1*	20.1	45.1*	22.6	20.9*	16.3

Drug #320. Hydrocodeinone hydrochloride

0.05	1.134	8	104.3	7.4	107.8a	9.3	114.4	14.9	105.5	8.1	101.9	16.9	103.5	10.5
0.1	1.435	4	102.8	9.2	115.3	3.4	99.5	11.1	91.5	14.5	106.0	9.6	94.3	20.6
0.3	1.855	7	101.7	9.4	126.6	36.6	100.1	5.6	103.4	8.9	104.7	12.1	106.3	10.3
0.5	2.134	4	104.5	7.1	102.0	26.4	100.8	3.6	98.8	17.3	105.3	3.6	99.3	20.1
1.0	2.435	8	100.9	6.6	111.9	25.8	102.6	5.1	99.8	6.1	105.0	8.2	101.9	7.9
3.0	2.855	6	101.8	8.9	112.7	23.1	109.5	32.9	94.8	13.4	108.2	23.6	91.2	12.2

a: n = 4

Drug #322. Dihydrohydrocodeinone hydrochloride (= Eucodal)

0.01	.454	8	103.4	8.4	109.5	12.4	102.6	10.9	104.5	12.5	106.0	14.8	107.9	16.2
0.02	.755	7	104.0	10.3	104.7	11.0	95.4	6.7	95.9	4.9	109.1	15.1	97.6	10.5
0.05	1.153	8	97.0	16.7	104.9	8.9	95.4	15.1	96.4	7.7	108.3	12.3	98.8	8.9
0.1	1.454	10	97.4	5.4	104.6	4.6	80.4*	10.2	91.5*	7.6	96.7*	5.1	92.1*	14.4
0.3	1.931	7	95.7	6.7	103.3	5.3	85.0*	13.7	82.7*	11.7	90.4*	8.9	76.9*	8.1
0.5	2.153	6	88.2	9.7	99.0	6.3	65.2*	16.2	74.7*	8.3	76.7*	7.0	69.7*	6.1
1.0	2.454	9	85.5	12.1	106.3a	14.9	60.2*	8.0	58.3*	7.8	72.3*	12.7	53.8*	9.5
3.0	2.931	7	64.9	19.6	93.9	10.9	30.4*	8.6	25.7*	15.7	46.9*	7.3	23.3*	11.4
5.0	3.153	4	57.5	6.4	100.0	10.7	24.0*	4.8	22.3*	7.0	38.0*	7.5	22.5*	6.7
10.0	3.454	3	64.7	13.0	85.0	7.6	16.3*	2.5	6.0*	1.4	37.7*	9.1	7.3	1.0

a: n = 7







Drug #370. Dihydrohydroxycodeine-C

0.1	1.498	8	101.8	12.5	102.6	14.3	98.0	21.8	99.0	13.2	104.9	11.6	103.0	24.4
0.2	1.800	8	98.1	7.3	97.3	13.5	96.9	7.9	100.5	14.1	109.3	17.7	106.0	17.6
0.5	2.198	8	101.5	8.6	109.0	13.2	99.0*	12.1	96.3*	8.1	111.1*	16.7	99.1*	7.0
1.0	2.498	7	105.4	6.9	103.6	8.0	87.0*	5.5	87.5a*	7.2	97.0*	6.9	86.5a*	7.8
3.0	2.976	8	100.6	6.4	93.9	9.1	72.0*	9.7	69.0*	8.0	78.8*	11.7	64.4*	13.3
5.0	3.198	6	95.7	11.2	98.7	12.3	62.3*	12.6	61.8*	10.8	68.3*	10.1	53.8*	12.2
10.0	3.498	7	90.7	14.2	99.1	18.6	51.9*	13.1	51.9*	11.7	62.6*	10.1	45.1*	12.6
20.0	3.800	4	83.8	5.2	78.5	17.3	45.3*	21.2	44.3*	27.0	47.8*	6.7	31.5*	20.4

a: n = 8

Drug #31. Bromocodineone

0.05	1.393	4	95.8	5.7	107.3	14.4	87.5	17.4	95.8	8.6	93.8	10.1	91.8	4.8
0.1	1.694	4	101.5	9.2	102.8	8.7	102.5	5.8	96.5	4.7	105.5	2.1	95.5	7.6
0.2	1.995	4	95.8	1.7	116.5	9.2	98.0	7.8	97.5	10.3	98.8	15.4	91.8	15.5
0.5	2.393	4	98.5	12.1	115.0	7.3	106.0	25.8	103.0	13.0	103.5	9.2	103.8	9.8
1.0	2.694	4	97.0	5.5	111.0	14.8	94.3	14.1	91.0	12.3	98.3	9.3	84.3	6.1

\* Effects used in calculating the equations given in table 3, and the straight lines that appear in figures 2 and 3.

† The data in columns 2 and 8 are plotted in figure 2; those in columns 2 and 12, in figure 3.



Comparison of drugs is more compact, however, if the potency of each is represented by a single index instead of by two numbers. A convenient value, used in two earlier papers, is the "threshold" dose predicted by the equation.

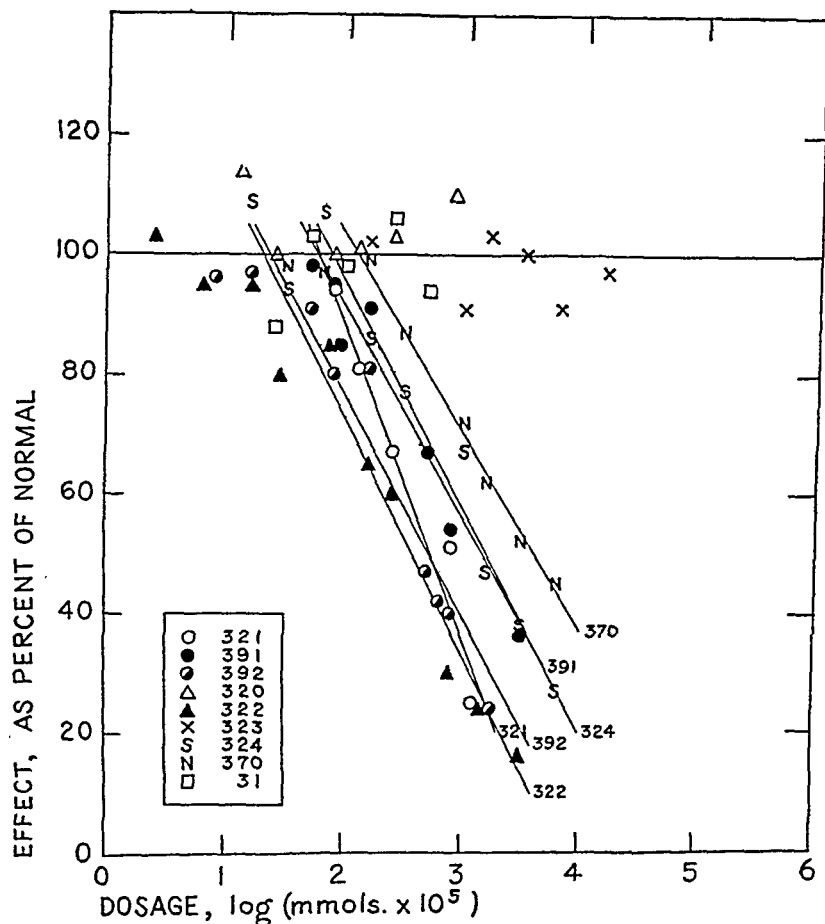


FIG. 2. GRAPH OF DOSE:RATE

Mean effects upon the respiratory rate of rabbits breathing air, as shown in table 2, have been plotted against dosage. The straight lines fitted to the points correspond to equations given in table 3.

The phrase has an arbitrary meaning rather than its usual one. Graphically, it is the dosage at which the line intersects the axis of 100 per cent, representing normal respiration. As can be seen in the figures, the lowest doses which experimentally caused slight depression are occasionally lower on the



dosage scale than this theoretical threshold. When the data trace the typical sigmoid curve which leaves the axis of normalcy asymptotically, there is no such thing as a true threshold. But this value which we call "threshold" is governed not only by the position of the S on the scale of dosage, but also

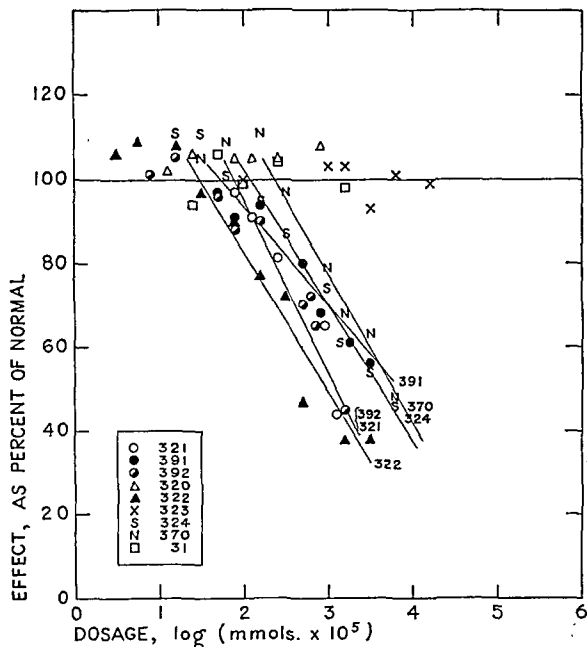


FIG. 3. GRAPH OF DOSE: MINUTE-VOLUME, LIKE THAT OF DOSE:RATE IN FIG. 2

by its steepness; and is as convenient and comprehensive as any index of potency that we know of, which can be calculated from the dosage-effect relationship. The straight lines and their equations were calculated chiefly in order to arrive at this figure. Table 3 gives it as "T," in milligrams, in millimoles, and as the logarithms of these. In the discussion that follows,



TABLE 3

*Linear equations\* to describe dosage-effect relationships*

RESPIRATORY FUNCTION	CONSTANTS							
	m	b		T				$\sigma$
		For dose in mgm.	For dose in mmoles. $\times 10^3$	Mgm.	Log mgm.	[mmoles. $\times 10^3$ ]	Log (mmoles. $\times 10^3$ )	Per cent
Drug #321. Acetylhydroxycodone hydrochloride								
Rate air.....	52.0	64.8	192.2	.21	-.677	59	1.772	11.5
Rate CO <sub>2</sub> .....	50.8	69.0	193.6	.25	-.610	69	1.839	13.1
M.V. air.....	41.1	76.4	177.0	.27	-.574	75	1.875	10.6
M.V. CO <sub>2</sub> .....	55.9	66.6	203.5	.25	-.598	71	1.851	12.0
Drug #391. Diacetyldihydrohydroxycodone-B acid tartrate								
Rate air.....	34.9	76.8	160.5	.22	-.665	54	1.732	11.9
Rate CO <sub>2</sub> .....	30.1	82.0	154.2	.25	-.599	63	1.798	10.2
M.V. air.....	23.9	83.9	141.2	.21	-.674	53	1.723	10.1
M.V. CO <sub>2</sub> .....	30.8	79.8	153.7	.22	-.656	55	1.741	12.5
Drug #392. Diacetyldihydrohydroxycodone-C acid tartrate								
Rate air.....	37.9	61.4	152.2	.10	-1.019	24	1.378	14.1
Rate CO <sub>2</sub> .....	36.4	68.6	155.8	.14	-.862	34	1.535	13.8
M.V. air.....	24.8	76.3	135.8	.11	-.955	28	1.443	13.2
M.V. CO <sub>2</sub> .....	36.4	66.0	153.3	.12	-.933	29	1.464	13.3
Drug #322. Dihydrohydroxycodone hydrochloride								
Rate air.....	39.5	53.0	151.7	.06	-1.192	20	1.311	11.2
Rate CO <sub>2</sub> .....	47.0	52.3	169.9	.10	-1.015	31	1.497	10.5
M.V. air.....	33.6	65.7	149.8	.10	-1.021	30	1.481	9.4
M.V. CO <sub>2</sub> .....	44.7	49.3	161.2	.07	-1.134	23	1.368	8.9
Drug #324. Dihydrohydroxycodone-B								
Rate air.....	37.7	76.6	170.7	.24	-.623	75	1.877	12.5
Rate CO <sub>2</sub> .....	37.0	82.3	174.7	.33	-.479	105	2.021	10.0
M.V. air.....	32.1	86.3	166.5	.37	-.428	118	2.071	7.8
M.V. CO <sub>2</sub> .....	40.4	80.6	181.5	.33	-.482	104	2.017	9.4
Drug #370. Dihydrohydroxycodone-C								
Rate air.....	32.8	86.3	168.2	.38	-.419	120	2.080	11.1
Rate CO <sub>2</sub> .....	33.6	86.1	170.1	.39	-.415	121	2.084	11.3
M.V. air.....	35.4	95.5	183.9	.75	-.127	235	2.372	9.4
M.V. CO <sub>2</sub> .....	42.2	85.7	191.1	.46	-.340	144	2.159	11.3

\* They were calculated from data given in table 2. The straight lines corresponding to these equations are drawn in figures 2 and 3. The equations have the form,  $Y = -mX + b$ , where

$Y$  = predicted effect, as per cent of the normal, at any given dose

$X$  = log dose in mgm. of base, or mmoles, per kilogram

$m$  = slope of the line, representing change in respiration (expressed as a per cent) for each unit of increase in log dose

$b$  = effect, as per cent of normal, when log dose is 0. It has no pharmacological meaning when its value is more than 100 per cent



the logarithm of the "threshold" dose in millimoles for depressing the minute volume of rabbits breathing air will be used as the criterion by which drugs are judged for purposes of comparison.

*T* has two major disadvantages as a measure of potency. a) How big the difference between one *T* and another must be to be significant we can not calculate by any rigid standard because one's choice of the data to include in the calculation enters into the uncertainty. We guess that a significant difference is at least 0.2 logarithmic units. b) *T*, like all abstracts, fails to include a great many things that are probably also important. When the drugs are compared according to a different criterion, such as the dosage sufficient to reduce rate or minute volume to half of the normal, many minor differences among them shift in magnitude or even in direction.

#### COMPARISON AND DISCUSSION

Table 4 arrays the drugs according to the molecular types (rows) and the kind of substituent at carbon-14 (columns) from which they are built, and assembles against this framework the values of their threshold doses. Such an arrangement makes clear the chemical relationships of the compounds, but leaves the pharmacological ones relatively obscure. All the possible pharmacological comparisons can be made at a glance, however, in table 5, where the same information is arranged to show chemical types against a numerical background.

The question posed was whether the hydroxyl group attached at carbon-14 in the molecule of morphine is unique among radicals, and consistent in its influence upon respiratory depression, as it is at carbon-6 and, in a different way, at carbon-3. The answer seems plain that it is not. As table 5 shows, one drug (dihydrohydroxycodine-B, #324) containing it is more potent than the corresponding form (codeine, #20) with hydrogen, one is less potent (dihydrohydroxycodine-C, #370, vs. dihydroisocodeine, #51), a third is nearly the same (dihydrohydroxycodineone, #322, vs. dihydrocodeinone, #154); and for each pair the ratio of dosages to produce a given effect is much less than tenfold. Two drugs (hydroxycodineone, #320 and dihydrohydroxycodine-A, #323), out of the five in which hydroxyl occurs, lack depressant power entirely, while one (dihydrohydroxycodineone, #322) is about as strong as the strongest compound in the set. In short, hydroxyl at carbon-14 is not consistently very different from other radicals.

In contrast, the acetoxyl group emerges from the three comparisons as superior in vigor to hydrogen in two cases and to hydroxyl in one. Within this limited set of drugs, then, acetoxyl, rather than hydroxyl, attached at carbon-14, is the unique group.

Shifting the ground of the discussion slightly, one may ask the effect of muzzling a hydroxyl group at carbon-14, instead of replacing it. With the hydroxyl at carbon-6, which is secondary-alcoholic in its nature, either pro-



# THE RESPIRATORY EFFECTS OF MORPHINE, CODEINE, AND RELATED SUBSTANCES

## IX. THE EFFECT OF MUZZLING THE PHENOLIC HYDROXYL<sup>1</sup>

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Received for publication April 19, 1941

For the ability of morphine to produce a number of characteristic physiological effects the phenolic hydroxyl group, on carbon-3, appears to be highly important (1). Codeine, in which a methyl radical has muzzled it, must be given in a tenfold dose to reduce the rate of a rabbit's breathing to the same extent. Whether the loss of power stems from tampering with the hydroxyl group *per se*, or from a special property of the methyl radical, is not known. The question calls for a study of the effects of drugs with a variety of groups substituted in the phenolic hydroxyl. Such a set of substances, chemically codeine-like, has been provided by the recent preparation of certain new compounds. The present paper reports how covering the phenolic hydroxyl group in morphine or analogous compounds, not only with methyl, but also with ethyl, methoxymethyl, benzyl, and dinitrophenyl, affects potency for depressing the respiration of rabbits.

### THE DRUGS

The seven drugs whose respiratory effects are to be described are listed in table 1. They were prepared in the laboratory of Lyndon F. Small at the University of Virginia, and came to us in the form of hydrochlorides, except benzylmorphine methyl ether which was an acid sulfate. A summary of the data concerning their physical properties, which Dr. Small furnished with the samples, is also given in table 1.

In this set of compounds, the hydrogen of the phenolic hydroxyl has been replaced by 2,4-dinitrophenyl, benzyl, methoxymethyl, or ethyl. The resulting group attached to carbon-3, as well as the methoxyl and hydroxyl groups with which they will be compared, are ranged at the left of the schematic molecule of morphine pictured in figure 1. Dinitrophenyl, benzyl, and ethyl muzzled hydroxyl in morphine itself, the resulting compounds being represented in the figure by the generalized structural formula when

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<sup>1</sup> The work reported in this paper is part of a unified effort by a number of groups to solve the problem of drug addiction. The participating organizations have been the Rockefeller Foundation, the National Research Council, the United States Public Health Service, the United States Bureau of Narcotics, the Massachusetts Department of Health, the University of Virginia, and the University of Michigan.



TABLE 1  
*Physical properties of the drugs*

DRUG	MOLECULAR WEIGHT	BASIC FRACTION per cent	MELTING POINT	APPROXIMATE SOLUBILITY IN WATER PER 100 OF H <sub>2</sub> O	OPTICAL ROTATION		
					[α] <sub>D</sub>	Tem-perature	°
			°C.			°C.	
463. 2,4-Dinitrophenylmorphine hydrochloride	487.81	92.5		0.5 at room temp.			
305. Benzylmorphine hydrochloride (Peronin)	429.87	87.4	280-281† with decomp.	0.6 at 20°	-55.4	23	0.813 H <sub>2</sub> O
306.* Benzyl dihydromorphine hydrochloride	431.89	87.4	233-235†	4 at 20°	-52.1	20	0.960 H <sub>2</sub> O
312.* Benzyl dihydrodesoxymorphine-D hydrochloride	397.68	90.9	249† with decomp.	1.1 at 23°	-34.4	23	1.060 alcohol
393.* Benzylmorphine 6-methyl ether acid sulfate	487.30	79.9	247-249†	1 at room temp. after heating to 40°	-90.1	25	0.910 H <sub>2</sub> O
316.* Methoxymethyl dihydromorphine hydrochloride	403.70	82.1	124-126 with decomp.	20 at 20°	-71.8	24	1.023 H <sub>2</sub> O
82. Ethylmorphine hydrochloride (Dionin)	367.66	85.2	124-125	14 at 15°			

\* New substances.

† *In vacuo*.



it is completed by the fragment marked A. The only drug in which the methoxymethyl radical was substituted was a dihydromorphine, corresponding to fragment B, a type of derivative of morphine in which benzyl, too, was tried. Lastly, we had a dihydrodesoxymorphine-D and a morphine-6-methyl ether, their special structures being indicated by fragments C and D, both with the benzyl group.

A general description of the physiological effects of one of the set, dinitrophenylmorphine (#463), and a brief discussion of its structure in relation to its effects, appeared in another publication (2), but we give here for the first time the actual data upon which the respiratory section of that account was based.

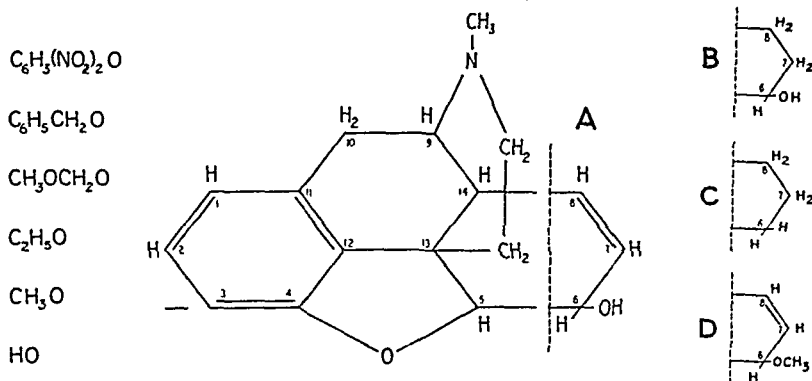


FIG. 1. THE STRUCTURAL FORMULAS OF THE DRUGS, DRAWN AS VARIANTS OF MORPHINE

They are represented here as alkaloid bases, but the samples used in the experiments were salts. For the names of the compounds see tables 1 and 4.

#### EXPERIMENTS AND RESULTS

The method used in the experiments is a routine procedure which has been described in the first paper of this series (3), and reviewed in the one immediately preceding this (4). The heart rate, consumption of oxygen, respiratory rate, and respiratory minute volume were measured in normal rabbits and in the same individuals an hour after injecting various doses of the drugs. Respiratory rate and minute volume were observed not only while the animals breathed room air, but also while they inhaled a mixture containing about 8 per cent  $CO_2$ . It is to be understood throughout this paper that every feature of a rabbit's behavior after administration of drug is expressed as a percentage of the normal; and that "dosage" signifies an amount per kilogram of animal.

The results are treated as in the preceding paper. A. Table 2 shows how all of the various functions measured responded on the average to different doses of the drugs. B. Figures 2 and 3 are composite graphs in which the data from table 2 for the respiratory rate and respiratory minute volume of rabbits breathing air have been plotted against dosage. C. Table 3 gives linear equations fitted by the method of least squares to the observations on



Mean effects upon heart rate and respiratory functions

DOSAGE		NUMBER OF ANIMALS	MEAN EFFECT											
Mgm.	Log (mmols. X 10 <sup>3</sup> )		Heart rate		Oxygen consumption		Air		CO <sub>2</sub>		Minute volume		CO <sub>2</sub>	
			Per cent	°	Per cent	°	Per cent	°	Per cent	°	Per cent	°		
Controls														
0.0		39	100.9	7.8	102.1	9.5	101.5	21.1	101.9	9.3	99.1	14.5	101.7	10.9
Drug #463. 2,4-Dinitrophenylmorphine hydrochloride														
0.02	0.613	8	99.8	1.8	99.8	10.4	98.4	14.8	99.4	16.3	102.0	11.0	101.1	18.3
0.05	1.011	6	94.3	20.4	101.3	8.9	93.8*	14.5	96.0*	13.5	100.2	12.3	99.8	20.8
0.10	1.312	7	99.9	11.0	104.7	10.6	82.4*	9.0	96.1*	10.3	100.1*	13.0	104.7*	14.8
0.25	1.710	8	100.4	8.5	94.3	8.9	88.4*	10.0	91.5*	9.3	95.5*	9.1	91.4*	12.4
0.5	2.011	6	96.2	9.2	98.7	9.8	65.2*	13.2	75.8*	13.2	82.3*	10.7	75.2*	13.8
1.0	2.312	7	106.6	7.8	101.9	10.6	71.1*	13.6	84.7*	12.6	90.4*	9.7	89.1*	14.0
2.0	2.613	7	96.6	7.3	95.0	19.8	72.4*	2.7	82.1*	5.1	93.6*	10.8	83.4*	6.6
5.0	3.011	10	94.4	18.9	93.3	12.6	58.8*	16.4	61.4*	18.7	73.4*	23.7	65.5*	27.6
10.0	3.312	8	92.1	12.2	97.6	13.3	62.5*	6.0	62.5*	10.3	74.9*	11.7	57.8*	14.9
15.0	3.488	4	94.0	8.8	97.0	5.6	54.3*	12.8	59.8*	7.5	68.8*	10.0	51.0*	14.8
20.0	3.613	4	74.8	12.3	81.8	5.5	45.0*	8.8	43.8*	7.0	68.5*	4.4	42.8*	5.6
Drug #305. Benzylmorphine hydrochloride														
0.1	1.367	8	101.4	7.6	104.8	8.9	102.1	14.5	102.0	14.0	102.8	7.7	101.9	16.9
0.2	1.668	8	98.1	5.3	107.9	7.8	95.8	14.5	102.0	20.5	104.1	11.2	102.9	20.7
0.5	2.066	8	96.5	6.9	101.9	7.3	95.6	9.9	104.0	13.8	101.9	15.9	102.1	13.1
1.0	2.367	8	100.8	7.0	108.3	5.3	99.9	21.9	98.6	14.3	114.1	13.8	99.9	10.4
3.0	2.844	10	103.4	6.1	107.5	11.3	90.1	11.2	93.1	5.2	101.5	11.5	89.6	5.1
5.0	3.066	10	103.8	12.5	113.4 <sup>a</sup>	12.9	93.9	8.0	96.3	10.2	92.5	6.3	89.3	10.5
10.0	3.367	7	99.3	10.5	109.0	13.0	87.9	11.3	85.8 <sup>b</sup>	4.4	88.6	4.4	84.3 <sup>b</sup>	0.5
20.0	3.668	6	118.2	17.1	114.0	17.2	116.0	14.7	112.0 <sup>c</sup>	27.2	93.2	12.8	90.4 <sup>c</sup>	12.1
a: n = 9    b: n = 6    c: n = 5														

a: n = 9 b: n = 6 c: n = 5

\* The effects of #463 used in calculating the equations given in table 3 and the straight lines appearing in figures 2 and 3.







Drug #393. Benzylmorphine 6-methyl ether acid sulfate

0.5	2.012	8	104.1	17.9	102.8	6.0	95.0	11.9	96.1	6.6	99.4	11.6	91.9	6.8
1.0	2.313	8	100.1	4.9	103.5	12.2	86.9	17.1	95.5	9.1	92.9	13.0	97.3	11.1
3.0	2.790	8	97.3	9.6	102.4	6.5	84.0	16.0	89.5	13.7	87.6	11.4	87.3	12.0
5.0	3.012	8	97.3	2.3	114.9	21.3	91.3	21.1	91.6	12.7	96.5	10.4	89.3	12.4
10.0	3.313	8	101.1	5.8	111.5	18.0	90.9	10.1	92.9	9.8	90.6	12.1	91.6	10.8
20.0	3.614	8	103.5	11.5	108.4	12.6	89.4	21.7	88.8	7.8	82.6	14.3	75.0	11.2
50.0	4.012	3	98.1	8.9			114.0	32.5			112.6	18.0	117.0	30.3

Drug #316. Methoxymethyldihydromorphine hydrochloride

0.05	1.093	8	107.0	11.3	107.0	10.5	101.0	16.4	102.0	7.6	116.1	21.9	103.6	6.9
0.1	1.394	8	97.9	6.0	102.0	10.6	96.8	20.4	100.5	12.3	103.5	13.2	103.9	18.4
0.2	1.695	8	100.8	2.7	105.3	9.2	88.3	14.1	94.9	4.8	95.0	5.4	97.6	6.5
0.5	2.093	8	102.5	7.3	112.9	25.0	95.4	12.1	89.0	12.0	98.6	18.0	83.5	12.4
1.0	2.394	18	115.4	16.7	104.6a	14.9	95.4	8.7	92.5	10.7	102.0	7.6	96.3	11.7
3.0	2.871	6	112.8	14.7	106.8	8.3	85.5	14.1	86.2b	7.3	86.0	9.0	82.8b	10.2
5.0	3.093	7	103.9	6.2	98.3	8.2	80.0	16.2	84.0	10.7	85.2	9.5	82.0	9.0
0.0	3.394	11	107.7	12.5	105.1c	8.6	74.0	12.1	75.5	7.5	82.0	11.0	75.7	7.7
0.0	3.695	11	100.7	6.7	108.0	15.5	64.2	8.8	60.9d	7.9	74.9	6.9	59.4d	7.2

a: n = 16 b: n = 5 c: n = 7 d: n = 7

Drug #82. Ethylmorphine hydrochloride (Dionin)

0.5	2.133	8	103.9	4.0	111.6	16.3	101.5	15.4	100.0	7.2	105.1	5.9	105.0	10.1
1.0	2.434	8	103.5	9.1	104.8	8.8	81.9	10.7	88.6	7.1	95.6	8.5	88.3	10.6
3.0	2.912	7	104.7	9.2	114.7	12.2	91.9	8.8	87.4	7.7	100.7	18.7	89.4	9.6
5.0	3.133	11	101.7	6.8	113.2	19.6	87.6	10.8	86.6	5.8	89.3	9.5	86.0	7.6
0.0	3.434	10	104.2	6.3	103.9	15.5	85.0	10.5	84.0	6.1	89.0	12.8	82.0	6.8
0.0	3.736	6	85.5	13.7	110.2	6.3	76.7	17.8	68.0	19.0	85.5	14.2	67.0	23.6



rate and minute volume, both on air and on CO<sub>2</sub>, after dinitrophenylmorphine (#463), the only drug of the group which caused effects consistent enough in their trend to be so treated. The data in table 2 utilized for calculating these equations are indicated by asterisks. The lines corresponding to those for rate and minute volume on air appear in figures 2 and 3. Table 3 gives the "threshold" doses predicted by the equations.

The erratic results caused by the six drugs other than dinitrophenylmorphine (#463) made practically impossible the choice of uncomplicated observations suitable for use in calculating equations. We have, however,

TABLE 3

*Linear equations\* which relate dosage to the effects upon four respiratory functions*  
Drug #463. 2,4-Dinitrophenylmorphine hydrochloride

RESPIRATORY FUNCTION	CONSTANTS							
	m	b		T				$\sigma$
		For dose in mgm. of base	For dose in mmols.	Mgm. base	Log mgm.	mmols. $\times 10^4$	Log (mmols. $\times 10^4$ )	
	per cent	per cent					per cent	
Rate air.....	15.2	71.3	107.0	.013	-1.884	2.9	0.461	12.1
Rate CO <sub>2</sub> .....	17.8	77.5	119.1	.054	-1.268	12	1.078	12.5
M.V. air.....	13.4	86.6	117.9	.099	-1.006	22	1.340	13.7
M.V. CO <sub>2</sub> .....	22.9	79.9	133.6	.013	-1.877	29	1.468	16.4

\* These equations were calculated from data in table 2. They have the form  $Y = -mX + b$ , where

$Y$  = predicted effect, as per cent of the normal, at any given dose

$X$  = log dose, in mgm. of base, or mmols., per kgm.

$m$  = slope of the line, representing change in respiration (expressed as a per cent) for each unit of increase in log dose

$b$  = effect, as per cent of normal, when log dose is 0. It has no pharmacological meaning when its value is more than 100 per cent

drawn by eye the lines which seemed best to follow the trends of the effects upon minute volume, and have taken the dosage at which each line crossed the axis of normalcy (100 per cent) as a "threshold" dose useful in comparing the potency of the drugs in this set with one another and with related compounds. These uncalculated "threshold" doses have as arbitrary a meaning as the values obtained by calculation for more purely depressant drugs such as dinitrophenylmorphine.

From the three forms of presentation of the results the reader can glean what information he chooses. In the discussion, we will comment on general properties of the drugs as shown in the graphs (figures 2 and 3), and then take the "threshold" doses as a basis for detailed comparisons.



## DISCUSSION

Dinitrophenylmorphine (#463) stands apart from the rest of this group (see figs. 2 and 3). Its effects varied with dosage in a way systematic enough

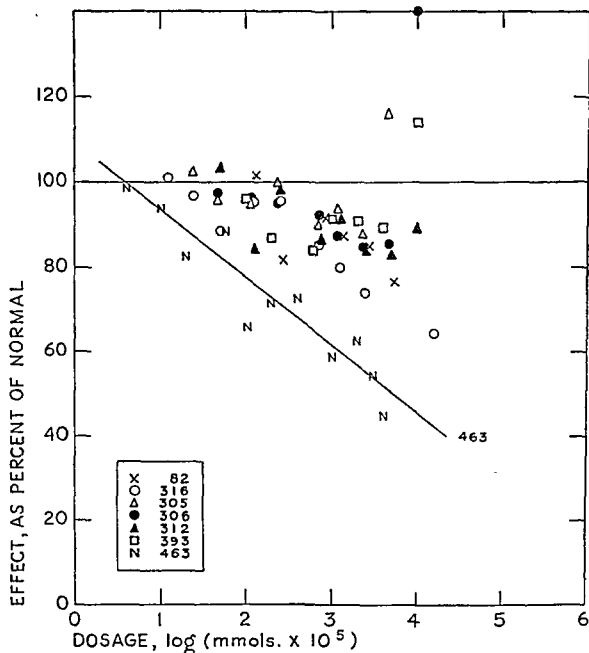


FIG. 2. GRAPH OF DOSE:RATE

Mean effects upon the respiratory rate of rabbits breathing air, as given in table 2, have been plotted against dosage. The straight line fitted to the points for drug #463 is described by an equation given in table 3. Results of the other compounds were too erratic for this treatment.

to be described conveniently by equations, as already mentioned. The lowest respiratory rate and minute volume reached with it were considerably lower than those achieved with the other drugs. Any degree of effect produced by



one of the others could be equalled with dinitrophenylmorphine in a smaller dose. Compared with codeine, it was more depressant at all though the disparity diminished with increasing doses. Compare

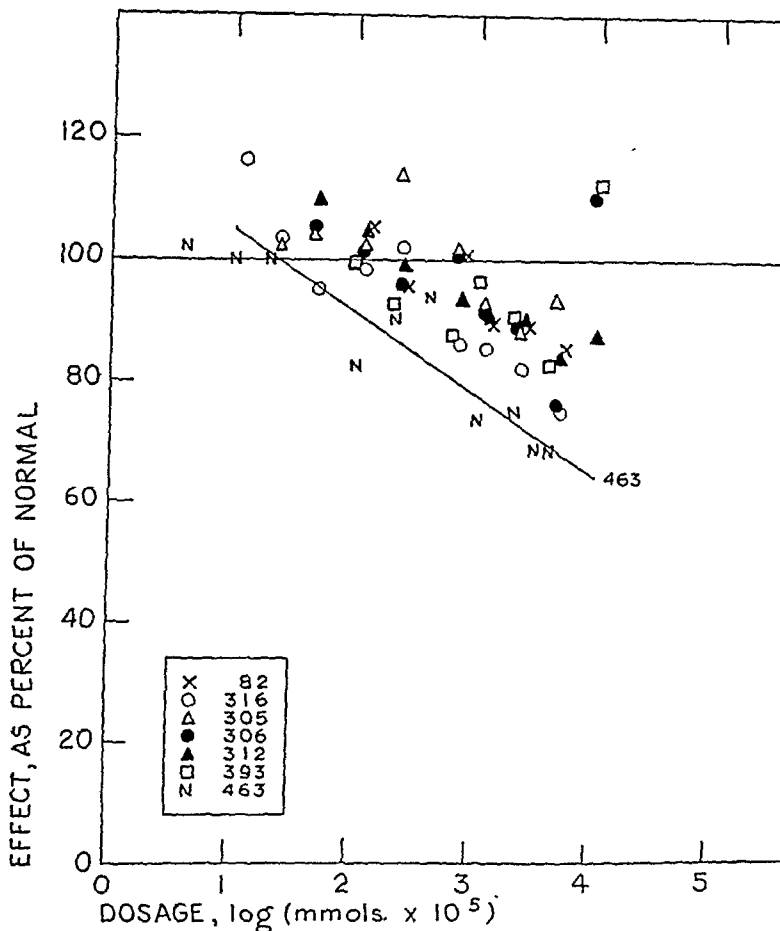


FIG. 3. GRAPH OF DOSE: MINUTE-VOLUME, LIKE THAT OF DOSE:RATE IN FIG

morphine, it was more depressant in barely effective doses, less depressant in doses larger than about 0.3 mgm.

The reduction caused in the rate and minute volume of respiration by other drugs of the set was small. After the most effective doses of benzylmorphine (#295), buprenorphine (#306), benzylhydromorphone (#307), and buprenorphine (#308), the rate and minute volume of respiration were reduced by 20-30% and 10-20%, respectively, compared to the control.



rate remained higher than 80 per cent of normal. Mean minute volume at only one point went below 80 per cent. Ethylmorphine (#82) was slightly more depressant than these benzyl derivatives, and methoxymethylmorphine (#316) still more so, but both traced dosage-effect curves that lie closer to the rest of the group than to dinitrophenylmorphine. It might be thought that if we had given still larger doses we would have obtained still larger

TABLE 4

*"Threshold" doses\* for depressing respiratory minute volume, arranged according to nuclear type and the group attached to carbon-3*

GROUP ATTACHED TO CARBON-3	NUCLEAR TYPE			
	A	B	C	D
	Morphine	Dihydromorphine	Dihydrodesoxy- morphine-D	Morphine 6-methyl ether
$C_6H_3(NO_2)_2O$	2,4-Dinitro- 0.10 phenylmor- (1.34) phine #463			
$C_6H_5CH_2O$	Benzylmor- 1.3 phine #305 (2.57)	Benzyl dihy- 0.77 dromor- (2.31) phine #306	Benzyl dihy- 0.90 drodesoxy (2.41) morphine-D #312	Benzylmor- 0.49 phine 6-meth- (2.10) yl ether #393
$CH_3OCH_2O$		Methoxy- 0.34 methyl di- (2.01) hydromor- phine #316		
$C_2H_5O$	Ethylmor- 0.63 phine #82 (2.30)			
$CH_3O$	Codeine† #2 1.3 (2.64)	Dihydrocode- 0.02 ine† #20 (2.43)	Dihydrode- 0.03 soxycode- (1.45) ine-D† #16	Codeine 0.50 methyl ether† (2.20) #85
HO	Morphine† #1 0.15 (1.72)	Dihydromor- 0.11 phine† #37 (1.53)	Dihydrode- 0.01 soxymor- (0.64) phine-D† #126	Morphine 6- 0.02 methyl eth- (0.73) er† #111

\* The doses are expressed in milligrams, and also (in parentheses) as 5 plus the logarithm of the number of millimoles given per kilogram.

† A drug whose effects have been described previously.

effects. With the four benzyl derivatives the results as they appear in the graphs bear witness that this is not so: the largest doses administered caused less depression than somewhat smaller doses, and in some instances were actually stimulating. With ethylmorphine and methoxymethylmorphine, the largest doses made some of the rabbits noticeably active, and thus led us to expect that giving more drug would increase activity sufficiently to offset the respiratory depression at least in part. Morphine, codeine, and many of their derivatives, when given in large amounts are not purely depressant



for respiration. The unusual feature of the present group of drugs is the slowness of depression achieved before the counter-effect sets in.

Table 4 shows the set of seven drugs arrayed in rows and columns which indicate chemical relationships. With them are eight analogous compounds whose effects are already published (5, 6, 7, 8). Beside each compound stands its "threshold" dose for depressing the minute volume of rabbits breathing air. This value is expressed both in milligrams, as in earlier papers, and also as the logarithm of the number of millimoles, to relate it to figures 2 and 3. It was estimated graphically in six instances (#305, #306, #312, #393, #316, #82) as has been said, but in the rest calculated from the equation describing the data. According to this criterion, dinitrophenylmorphine (#463) is more potent than any analogous drug, benzylmorphine (#305), ethylmorphine (#82), codeine (#2), or morphine itself (#1). As a compound in which the phenolic hydroxyl group has been muzzled, it is unique among them in this respect; no other codeine-like drug in the table excels its morphine-like counterpart. Morphine itself is about ten times as strong as its benzyl, ethyl, and methyl derivatives, which do not differ much among themselves. Likewise, dihydromorphine is ten times as strong as its methyl, methoxymethyl, or benzyl derivatives, which are much alike. Dihydrodesoxymorphine-D and morphine-6-methyl ether are also superior to their derivatives, but in a less regular way. The latter is fully twelve times as depressant as its two derivatives which are almost exactly equal; while the dihydrodesoxy compounds are the only ones in which a distinct difference appears between the benzyl and methyl radicals, the latter producing the more depressant drug. Thus it appears that in only two of the pairs of compounds now available for this type of comparison (#463 vs. #1; #312, and #16, vs. #126) has the nature of the group substituted for hydroxyl at carbon-3 influenced to any significant extent the degree of weakening which substitution at this point nearly always causes.

It should be added that the "threshold" dose shows no correlation with any of the known physical properties of these drugs, such as melting point, optical rotation, and solubility (consult table 1).

#### SUMMARY

The depressant effects upon rabbits' respiration are given for 7 derivatives of morphine in which the phenolic hydroxyl group, at carbon-3, has been muzzled by ethyl, methoxymethyl, benzyl, or dinitrophenyl. Using the "threshold" dose for depressing minute volume as a criterion of potency, we have compared these and 8 related drugs whose effects have been previously described, to ascertain whether the loss of potency associated with substitution at that point depends upon the intactness of the phenolic hydroxyl radical *per se*, or upon the special properties of the substituent. Dinitrophenylmorphine was stronger than morphine in small doses though weaker in doses



larger than about 0.3 mgm. The remaining codeine-like compounds were all weaker than their morphine-like analogs. Among them, it seemed to make little difference whether the substituent was methyl, ethyl, methoxymethyl, or benzyl, except in one instance, when methyl (in dihydridesoxycodine-D, #16) proved much more depressant than benzyl (in benzyldihydridesoxymorphine-D, #312).

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- (1) SMALL AND EDDY: U. S. Pub. Health Rep., Suppl. no. 138, 1938.
- (2) EDDY AND SUMWALT: THIS JOURNAL, 67: 127, 1939.
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- (4) SUMWALT AND OSWALD: *Ibid.*, 73: 229, 1941.
- (5) WRIGHT: *Ibid.*, 61: 343, 1934.
- (6) WRIGHT AND BARBOUR: *Ibid.*, 63: 34, 1935.
- (7) WRIGHT AND BARBOUR: *Ibid.*, 61: 440, 1937.
- (8) SUMWALT, OSWALD AND LUSK: *Ibid.*, 73: 274, 1941.



# THE RESPIRATORY EFFECTS OF MORPHINE, CODEINE, AND RELATED SUBSTANCES

## X. THE EFFECT OF SUBSTITUTING AN ADDITIONAL GROUP IN RING-THREE<sup>1</sup>

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Received for publication April 19, 1941

In studies of the relation between chemical structure and physiological activity among derivatives of morphine, no chemical change, short of breaking the nucleus itself, has been found to influence more strongly the depressant effect upon respiration than substitutions for the secondary alcoholic hydroxyl group at carbon-6 (1, 2, 3, 4, 5), or interchange of groups between carbon-6 and carbon-8 (6, 7, 8). It is interesting, therefore, to see whether another type of variation in the same region of the molecule is also influential. We present in this paper the respiratory effects of ten drugs characterized by the presence of an extra radical in ring-III. Whether it is attached at carbon-5 or carbon-7 there is not sufficient chemical evidence to determine, but the attachment is believed to be the same in all of the compounds, so that they may be compared profitably as a set with corresponding ones in which no such additional radical has been inserted. The effects of an eleventh drug, lacking the extra group, are also given, because the background of unsubstituted analogs whose effects were already known was incomplete without it. The outcome of the study shows that in ring-III additional substitution alters depressant strength sometimes in one direction, sometimes in another, and by variable amounts. The specific properties of the different radicals can not be entirely accountable for the variability, because a methyl group in one case increased potency, in three cases decreased it, and in one case made no difference at all. In short, the results are so inconsistent among themselves as to baffle explanation, but they accord with the familiar concept that the chemistry of this portion of the nucleus is important for the pharmacology of morphine.

<sup>1</sup> The work reported in this paper is part of a unified effort by a number of groups to solve the problem of drug addiction. The participating organizations have been the Rockefeller Foundation, the National Research Council, the United States Public Health Service, the United States Bureau of Narcotics, the Massachusetts Department of Health, the University of Virginia, and the University of Michigan.



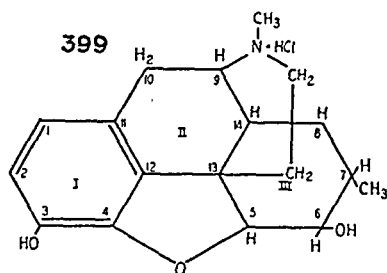
## THE DRUGS

The compounds were made in the laboratory of Lyndon F. Small at the University of Virginia, eight of them being new substances made there for the first time. The substituent in ring-III which is the focus of attention in this paper, was a methyl, ethyl, isopropyl, amyl, benzyl, or phenyl, group; while the compounds to which it was attached fell into the class of dihydromorphines, -morphinones, -codeines, -codeinones, or -codeinone enol acetates. Figure 1 shows the structural formulas of the drugs; and indicates, for one of them (#399), the nature of the chemical uncertainty which is equally characteristic of all. Table 1 lists them, and tells the state, whether base or salt, in which they were sent to us. For use, we dissolved the basic ones in an approximate equivalent of HCl. Table 1 also gives a summary of their physical properties, as determined in the Virginia laboratory.

## EXPERIMENTS AND RESULTS

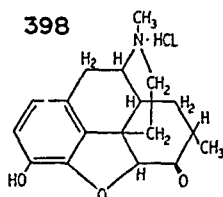
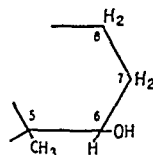
The experiments were conducted in a way that has already been described (6, 9). The consumption of oxygen, the respiratory rate, and the respiratory minute volume of rabbits breathing room air were measured before, and one hour after, the subcutaneous administration of various doses of the drugs; also the respiratory rate and volume of the animals while inhaling about 8 per cent CO<sub>2</sub>; and, incidentally, the heart rate. The post-drug rate, or minute volume, or other function, is expressed as a percentage of the normal. Results are set forth in three ways, discussed in an earlier paper of this series (9): *A*, a complete assemblage in table 2 of averages of all the effects observed; *B* and *C*, abstracts of the data in table 2. *B* consists of pictorial abstracts: figures 2, 3, and 4 repeat graphically the effects of the drugs upon the utilization of oxygen, the respiratory rate of rabbits breathing air, and the minute volume of rabbits breathing air. *C* is a mathematical abstract: table 3 gives the equations of the straight lines that fit the data for rate and minute volume best, according to the criterion of least squares. The values selected from table 2 for the calculation of these equations are indicated by asterisks, and the lines corresponding to those that deal with rabbits on room air have been traced in figures 3 and 4. First we will draw a few generalizations about the respiratory effects of the drugs from the plotted results in the graphs; then make a detailed comparison of their potency in relation to their chemistry. The measure of potency used is the "threshold" dose for depressing minute volume, a value given as *T* in table 3, and arrived at by extrapolating the straight lines. It has been calculated in milligrams, so that it can be compared readily with earlier work published in this series, and also in millimoles which for theoretical considerations constitute a fairer unit of comparison. Far more information can be drawn from tables 2 and 3, and from figures 2, 3, and 4, than can be discussed in the available space.



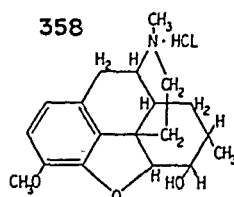


Methyldihydromorphine

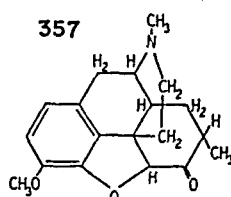
or



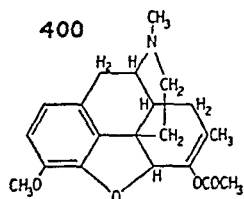
Methyldihydro-morphinone



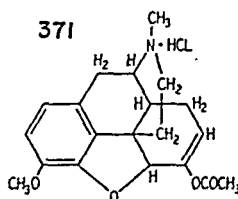
Methyldihydrocodeine



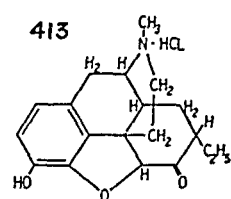
Methyldihydro-codeinone



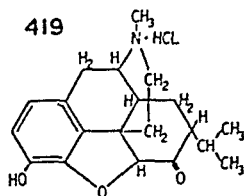
Methyldihydro-codeinone enol acetate



Dihydrocodeinone enol acetate



Ethyldihydro-morphinone

**423****440****441** $(\text{CH}_2)_4\text{CH}_3$  $\text{CH}_2\text{C}_6\text{H}_5$  $\text{C}_6\text{H}_5$ 

Isopropyl-, Amyl-, Benzyl-, Phenyl-dihydromorphinone

FIG. 1. STRUCTURAL FORMULAS OF THE DRUGS

The uncertainty indicated by the alternative arrangements for methyldihydromorphinone (#399), at the top of the page, exists for each of the other drugs also.



TABLE 1  
*Physical properties of the drugs*

DRUG	MOLECULAR WEIGHT	BARIC FRACTION	MELTING POINT	APPROXIMATE SOLUBILITY IN PARTS PER 100 OF H <sub>2</sub> O	TEMPERATURE OF SOLUBILITY TEST	OPTICAL ROTATION	
						[ $\alpha$ ] <sub>D</sub>	Tem- pera- ture
		per cent	°C.				°
399. Methylidihydromorphine hydrochloride	337.7	89.2	316-317* with decomp.	very		-64.9	1.001 water
398.† Methylidihydromorphinone hydrochloride	335.6	89.1	324-326* with decomp.	very		-99.7	1.013 water
358†. Methylidihydrocodeine hydrochloride	351.7	89.6	283-283.5*	very		-68.1	0.977 water
357†. Methylidihydrocodeinone	313.2	100	144-145	salts very soluble		-147.2	0.992 alc.
400. Methylidihydrocodeinone enol acetate (Methylacedicon)	355.2	100	193-194.5	soluble in HCl		-143.6	0.982 alc.
371. Dihydrocodeinone enol acetate hydrochloride (Acetidcon)	377.7	90.3	131.5-134	very		-130	1.4 water
413.† Ethylidihydromorphinone hydrochloride	349.6	89.6	Amorphous	very		-62.8	1.019 water
419.† Isopropylidihydromorphinone hydrochloride	363.7	90.0	340-341* with decomp.	5		-64.2	0.873 water
423.† Amylidihydromorphinone hydrochloride	391.7	90.7	322-325* with decomp.	1.5	20°	-63.9	1.03 water
440†. Benzylidihydromorphinone hydrochloride	411.7	91.2	241-242*	0.6	room temp.	-100.6	0.298 water
441†. Phenylidihydromorphinone hydrochloride	397.7	90.8	334-337* with decomp.	1	room temp.	-126.9	0.528 water

\* In vacuo.

† New drugs.







Drug #358. Methylidihydrocodeine hydrochloride

0.3	3.931	8	98.1	6.5	107.1	6.6	117.4	40.8	107.6	19.7	109.1	21.9	106.6	18.7
0.5	4.153	7	101.4	8.9	98.6	9.5	89.6	20.4	93.6	8.1	93.0	11.5	95.1	11.1
1.0	4.454	8	96.6	4.6	105.6	7.5	97.6	20.8	89.8 <sup>a</sup>	8.8	90.3	8.4	90.0 <sup>a</sup>	7.5
3.0	4.931	6	97.5	5.1	97.7	12.6	102.5	20.3	93.0	6.8	93.5	18.2	89.2	9.2
5.0	5.153	4	99.3	4.9	105.5	4.7	92.8	20.0	94.3	9.8	100.5	11.4	88.5	5.1
5.7	5.210	4	96.5	5.0	101.5	6.9	102.8	12.6	107.5	9.5	99.8	8.7	115.3	10.7
10.0	5.454	8	98.6	8.6	96.9	6.1	92.6	11.2	95.0	5.2	100.6	5.9	93.0	6.2

a: n = 4

Drug #357. Methylidihydrocodeinone

0.3	3.981	8	96.6	8.2	106.9	13.0	92.3*	7.0	93.4*	12.5	96.6*	13.5	91.3*	14.1
0.5	4.203	8	100.3	6.0	101.6	11.8	86.9*	10.6	85.0*	5.4	94.5*	9.3	83.0*	11.9
1.0	4.504	8	94.1	5.0	102.9	10.7	81.1*	7.5	82.8*	5.8	95.1*	9.5	82.5*	8.9
3.0	4.981	8	91.5	6.7	102.9	22.8	79.9*	25.6	74.9*	9.9	85.5*	15.7	70.0*	11.1
5.0	5.203	8	87.9	7.8	95.0	16.3	66.3*	14.9	70.0*	7.4	84.0*	13.7	66.0*	11.0
10.0	5.504	8	83.1	16.8	117.1	27.6	76.4	19.8	80.3	19.6	102.3	31.6	182.9	25.8

Drug #400. Methylidihydrocodeinone enol acetate

0.2	3.751	8	98.4	4.2	99.3	9.4	90.6*	10.2	95.5*	8.9	95.4*	5.0	93.9*	7.4
0.5	4.149	8	98.4	7.1	106.8	10.1	78.4*	16.0	89.0*	4.5	86.1*	17.2	93.8*	26.0
1.0	4.449	8	99.4	8.4	98.1	11.4	77.6*	14.6	88.1*	8.6	86.4*	10.8	83.3*	9.8
2.0	4.751	8	100.8	7.4	97.9	5.5	85.3*	18.7	89.0*	11.2	85.4*	12.8	85.9*	14.9
5.0	5.149	7	86.4	12.3	90.4	9.0	66.0*	9.6	71.0*	11.1	74.4*	6.1	65.7*	10.1
10.0	5.449	8	84.5	21.2	107.9	23.0	77.9	26.3	69.8	12.1	89.1	17.8	62.0	10.7

\* Effects used in calculating the lines of figures 3 and 4 and table 3.

† The data from columns 2 and 6 are plotted in figure 2; from columns 2 and 8, in figure 3; from columns 2 and 12, in figure 4.



TABLE 2—Concluded

DOSEAGE		NUMBER OF ANIMALS	MEAN EFFECT											
Mgm.	Log (mmoles. × 10 <sup>3</sup> )		Heart rate		Oxygen consumption		Ratio				Minute volume			
			Per cent	σ	Per cent	σ	Air		CO <sub>2</sub>		Air		CO <sub>2</sub>	
							Per cent	σ	Per cent	σ	Per cent	σ	Per cent	σ
Drug #371. Dihydrocodeinone enol acetate hydrochloride														
0.1	3.423	8	96.8	5.7	101.1	7.3	94.1*	9.6	97.3*	7.2	99.0*	9.6	94.3*	9.2
0.2	3.724	8	97.4	9.9	99.3	12.1	83.4*	8.6	94.8*	10.6	92.3*	7.8	92.6*	8.0
0.5	4.122	8	94.0	5.9	92.9	8.2	74.9*	12.3	78.5*	9.7	88.4*	10.9	73.5*	8.2
1.0	4.423	8	91.4	8.8	96.5	9.9	62.8*	10.2	71.8*	5.9	78.4*	7.4	66.1*	7.4
2.0	4.724	8	83.9	17.6	87.9	9.2	51.3*	10.3	58.9*	18.2	63.8*	12.1	57.6*	23.1
5.0	5.122	8	61.6	12.0	83.1	12.5	38.4*	14.5	31.8*	18.4	52.3*	8.2	24.5	10.2
Drug #413. Ethyldihydromorphinone hydrochloride														
0.01	2.457	8	99.0	7.7	106.9	4.8	94.8	9.9	94.8	6.8	99.8	10.9	94.1	7.7
0.02	2.757	8	97.1	8.4	102.5	8.8	97.3	8.4	98.4	10.0	95.5	3.6	95.3	12.3
0.05	3.155	8	95.5	9.1	97.8	6.4	80.1*	19.5	88.4*	8.4	90.5*	14.3	88.5*	9.0
0.1	3.457	7	96.7	7.0	105.9	14.9	82.7*	14.9	84.0*	8.2	92.1*	14.4	80.9*	10.3
0.2	3.757	7	93.0	6.6	91.9	12.6	66.7*	16.9	72.3*	12.8	77.9*	12.2	69.0*	13.6
0.5	4.155	7	91.3	16.5	95.0	11.8	50.9*	15.5	53.3*	18.7	61.3*	11.8	43.9*	17.8
1.0	4.457	8	63.9	13.3	77.9	8.3	36.3*	14.6	35.1*	15.9	49.9*	13.1	28.8*	19.6
Drug #419. Isopropyldihydromorphinone hydrochloride														
0.05	3.138	8	98.6	5.3	107.7a	10.0	89.9*	14.5	92.6*	8.0	99.0*	8.9	90.0*	8.4
0.1	3.439	8	98.9	5.9	95.3	8.0	87.1*	14.9	93.9*	8.6	99.8*	18.4	92.4*	10.7
0.2	3.740	7	98.0	9.9	109.0	5.8	84.0*	8.1	80.0*	2.8	94.7*	7.0	78.9*	5.6
0.5	4.138	8	89.4	10.2	88.3		66.6*	4.1	66.8*	2.4	79.6*	1.9	60.9*	3.7
1.0	4.439	8	83.8	12.3	91.1	9.4	58.5*	9.4	58.1*	12.4	71.8*	7.8	47.9*	13.0
2.0	4.740	6	73.2	20.1	83.0	16.9	51.2*	14.9	51.7*	33.1	60.2*	17.6	46.0*	19.0

a: n = 7



Drug #423. Amyldihydromorphinone hydrochloride

	0.0001	0.407	11	96.8	5.4	98.6	9.7	96.5	7.0	98.3	4.6	99.6	4.9	99.3	8.7
0.0002	0.708	8	8	99.1	6.7	106.6	9.1	102.4	11.7	101.1	9.7	105.6	6.7	102.3	9.2
0.0005	1.106	8	8	96.9	2.9	105.0	11.5	95.0	6.6	98.4	2.7	95.4	9.3	96.3	6.5
0.001	1.407	8	8	100.0	5.5	109.0	11.3	88.9	16.4	99.9	10.2	99.6	7.6	98.9	13.2
0.002	1.708	8	8	96.1	6.7	103.4a	16.0	88.9*	7.7	88.8*	12.5	94.8	10.5	85.0	13.9
0.005	2.106	8	8	92.6	8.8	113.6	19.1	83.6*	5.6	78.3*	7.8	94.4*	8.5	74.1*	10.2
0.01	2.407	8	8	89.0	8.0	93.1	12.9	65.9*	15.1	73.1*	8.1	82.9*	11.2	78.3*	15.6
0.02	2.708	8	8	85.5	13.0	87.8	12.6	60.8*	19.6	61.8*	18.6	70.5*	19.5	58.1*	26.3
0.05	3.106	8	8	57.9	14.4	81.9	8.9	28.1*	8.2	23.0*	11.8	41.4*	7.6	20.9*	9.4
0.1	3.407	8	8	62.3	23.0	72.9	15.7	19.9*	9.5	17.1*	8.9	40.1*	12.3	16.4*	7.6
0.2	3.708	8	8	55.3	12.8	68.0	16.0	16.6	5.0	7.6	5.0	34.4	6.0	14.4	16.0
0.5	4.106	4	4	70.8	14.6	70.8	34.4	20.3	4.1	6.0	0.8	42.3	9.3	6.5	1.7

a: n = 7

Drug #440. Benzylidihydromorphinone hydrochloride

0.01	2.386	8	96.3	4.1	105.4	18.8	98.4	2.0	100.4	4.7	103.3	7.6	103.5	7.9
0.02	2.687	8	99.1	4.3	111.1	11.1	95.0	15.9	98.6	4.7	105.9	13.7	101.6	4.6
0.05	3.085	8	100.8	4.7	101.6	16.2	92.5*	15.0	96.0*	10.4	100.4*	10.6	97.3*	13.1
0.1	3.386	8	96.8	7.5	101.5	10.8	80.8*	11.4	81.4*	3.1	89.3*	6.0	78.4*	5.9
0.2	3.687	8	95.9	9.0	101.5	13.5	77.0*	8.6	81.3*	6.4	89.0*	4.2	77.3*	6.0
0.5	4.085	8	86.4	5.9	95.9	7.5	64.9*	13.5	67.9*	6.1	85.9*	19.8	60.5*	11.8
1.0	4.386	8	88.1	9.0	95.0	10.2	50.4*	15.5	61.9*	9.0	74.8*	11.0	57.0*	10.9
2.0	4.687	7	83.7	5.4	89.1	16.9	53.4	14.4	57.4	9.7	73.6	12.8	54.4	11.2

Drug #441. Phenylidihydromorphinone hydrochloride

0.01	2.401	8	101.3	5.0	104.5	4.9	100.3	9.1	99.8	18.8	102.8	9.6	98.9	23.7
0.02	2.701	8	99.0	5.1	97.5	6.8	92.5	8.1	99.3	6.6	95.8	4.2	99.3	8.0
0.05	3.099	8	101.3	8.5	96.5	9.6	87.1	11.3	96.8	13.2	93.3	13.5	101.4	18.3
0.1	3.401	8	96.5	11.1	104.9	6.8	89.6*	12.0	85.1*	6.5	97.3*	7.4	85.6*	12.0
0.2	3.701	8	98.6	7.1	107.1	10.7	84.0*	10.5	84.4*	6.9	94.9*	10.6	81.6*	7.2
0.5	4.099	8	93.3	9.1	98.1	14.2	62.8*	9.4	64.6*	12.2	74.8*	14.5	62.8*	17.7
1.0	4.401	7	81.3	21.8	93.0	11.1	46.4*	18.3	50.3*	18.4	62.4*	13.9	46.4*	18.0
2.0	4.701	8	63.4	16.0	73.9	7.9	28.4*	14.3	26.6*	14.1	44.4*	12.3	23.9*	10.6



TABLE 3  
*Linear equations\* to describe dosage-effect relationships*

RESPIRATORY FUNCTION	CONSTANTS							
	m	b		T				$\sigma$ Per cent
		For dose in mgm.	For dose in mmoles. $\times 10^3$	Mgm.	Log mgm.	mmoles. $\times 10^3$	Log (mmoles. $\times 10^3$ )	
Drug #399. Methyldihydromorphine hydrochloride								
Rate air.....	22.2	80.4	180.5	.13	-.887	4,310	3.635	15.0
Rate CO <sub>2</sub> .....	22.1	87.4	187.5	.27	-.568	8,980	3.953	9.5
M.V. air.....	40.3	107.5	289.5	1.54	+.186	51,000	4.708	9.6
M.V. CO <sub>2</sub> .....	36.3	96.3	260.4	.79	-.102	26,000	4.420	12.8
Drug #398. Methyldihydromorphinone hydrochloride								
Rate air.....	33.5	32.2	183.8	.0095	-2.023	316	2.500	14.6
Rate CO <sub>2</sub> .....	36.7	31.6	197.4	.0136	-1.866	455	2.658	12.9
M.V. air.....	27.4	47.7	171.8	.0124	-1.907	414	2.617	10.9
M.V. CO <sub>2</sub> .....	36.8	29.3	195.9	.0120	-1.920	402	2.604	12.5
Drug #357. Methyldihydrocodeinone								
Rate air.....	17.6	82.5	161.9	0.102	-0.992	3,250	3.512	14.8
Rate CO <sub>2</sub> .....	17.4	82.4	160.8	0.098	-1.010	3,120	3.494	9.1
M.V. air.....	11.0	91.9	141.3	0.183	-0.737	5,850	3.767	11.9
M.V. CO <sub>2</sub> .....	19.7	79.9	168.9	0.096	-1.016	3,080	3.483	10.8
Drug #400. Methyldihydrocodeinone enol acetate								
Rate air.....	12.7	80.4	136.9	0.029	-1.546	800	2.903	8.6
Rate CO <sub>2</sub> .....	14.4	86.7	150.6	0.118	-0.928	3,330	3.522	9.4
M.V. air.....	12.7	85.6	141.9	0.073	-1.138	2,050	3.311	11.6
M.V. CO <sub>2</sub> .....	18.6	84.6	167.4	0.149	-0.826	4,200	3.623	14.7
Drug #371. Dihydrocodeinone enol acetate hydrochloride								
Rate air.....	32.7	60.5	206.8	0.062	-1.205	1,830	3.262	10.5
Rate CO <sub>2</sub> .....	37.7	64.2	232.4	0.112	-0.951	3,280	3.516	12.8
M.V. air.....	27.9	73.1	197.2	0.108	-0.967	3,160	3.500	9.6
M.V. CO <sub>2</sub> .....	39.4	59.8	235.9	0.095	-1.020	2,800	3.447	13.0
Drug #413. Ethyldihydromorphinone hydrochloride								
Rate air.....	36.2	37.4	200.4	0.019	-1.730	594	2.774	16.1
Rate CO <sub>2</sub> .....	41.7	36.8	224.6	0.031	-1.516	973	2.988	13.2
M.V. air.....	27.8	53.1	178.5	0.021	-1.686	658	2.818	12.6
M.V. CO <sub>2</sub> .....	40.5	31.8	214.1	0.021	-1.685	660	2.819	13.2
Drug #419. Isopropylhydromorphinone hydrochloride								
Rate air.....	26.0	58.9	175.3	0.026	-1.591	783	2.894	12.1
Rate CO <sub>2</sub> .....	28.7	58.1	186.9	0.035	-1.458	1,070	3.027	10.6
M.V. air.....	30.7	69.4	207.0	0.101	-0.998	3,070	3.487	11.8
M.V. CO <sub>2</sub> .....	38.6	50.0	223.0	0.051	-1.296	1,550	3.189	12.4



TABLE 3—*Concluded*

RESPIRATORY FUNCTION	CONSTANTS							
	m	b		T				$\sigma$
		For dose in mgm.	For dose in mmoles. $\times 10^3$	Mgm.	Log mgm.	mmoles. $\times 10^3$	Log (mmoles. $\times 10^3$ )	Per cent
Drug #423. Amyldihydromorphinone hydrochloride								
Rate air... ..	43.9	24.5	170.8	0.0015	-2.836	41	1.613	12.9
Rate CO <sub>2</sub> .....	45.6	29.6	173.4	0.0014	-2.840	41	1.609	15.4
M.V. air.....	45.9	12.3	191.9	0.0036	-2.447	101	2.003	12.3
M.V. CO <sub>2</sub> ....	53.3	41.2	195.8	0.0022	-2.650	63	1.799	16.6
Drug #440. Benzyldihydromorphinone hydrochloride								
Rate air.....	24.9	57.5	167.6	0.020	-1.710	519	2.715	12.2
Rate CO <sub>2</sub> .....	24.8	60.3	170.0	0.025	-1.602	667	2.824	7.6
M.V. air.....	16.4	76.4	148.9	0.036	-1.443	962	2.983	11.4
M.V. CO <sub>2</sub> .....	29.8	53.2	185.2	0.027	-1.570	717	2.855	10.2
Drug #441. Phenylhydromorphinone hydrochloride								
Rate air.....	48.5	43.7	259.2	0.069	-1.161	1,910	3.282	12.6
Rate CO <sub>2</sub> .....	45.8	44.6	248.1	0.062	-1.209	1,710	3.233	12.7
M.V. air.....	41.9	58.7	245.0	0.104	-0.985	2,860	3.457	11.4
M.V. CO <sub>2</sub> .....	48.0	41.7	255.0	0.061	-1.214	1,690	3.228	13.6

\* They were calculated from data given in table 2. The straight lines corresponding to these equations are drawn in figures 3 and 4. The equations have the form,  $Y = -mX + b$ , where

$Y$  = predicted effect, as per cent of the normal, at any given dose

$X$  = log dose, in mgm. of base, or mmoles., per kilogram

$m$  = slope of the line, representing change in respiration (expressed as a per cent) for each unit of increase in log dose

$b$  = calculated effect, as per cent of normal, when log dose is 0. It has no pharmacological meaning when its value is more than 100 per cent.

M.V. stands for minute volume, T for "threshold" dose (dose for predicted effect of 100 per cent,  $\sigma$  for the standard deviation of the equation. The rounded figures for mmoles.  $\times 10^3$  are not accurate beyond two places.

#### DISCUSSION

Certain general statements are true of these drugs with respect to any of the respiratory functions that have been studied. The least depressant among them were the ones that are not morphinones: methyldihydrocodeine (#358), which seemed to have no depressant effects at all, and methyldihydromorphine (#399), methyldihydrocodeinone (#357), and methyldihydrocodeinone enol acetate (#400), which caused only slight changes, and those at high dosages. The most depressant among them was amyldihydromorphinone (#423). It was effective in the smallest dosages, its effect increased most rapidly with



increasing dosage, and it accomplished the most profound depression before causing restlessness to offset depression. This drug surpassed not only the other drugs of this set, but also all of the sixty-five derivatives of morphine

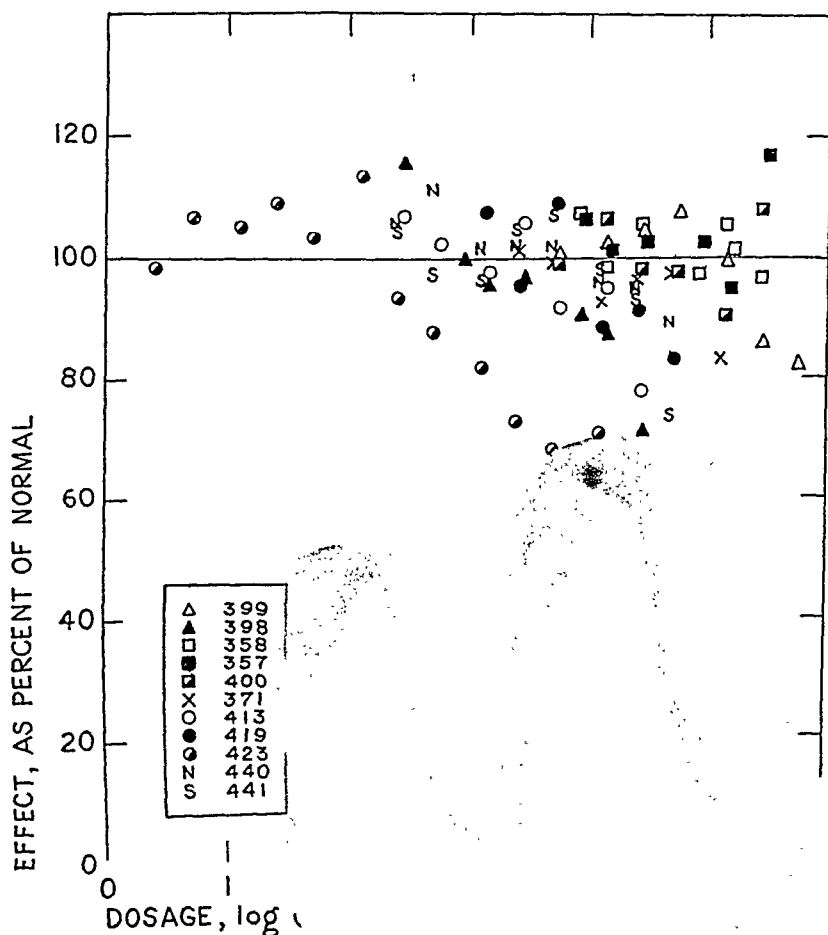


FIG. 2. THE MEAN EFFECTS

whose effects upon respiratory other radicals (in methyl-, #3 #440, and phenyl-dihydromc tion as amyl, in a molecule of potency. Therefore the distinct



sibly to some rearrangement occurring elsewhere in the molecule without the knowledge of the chemists.

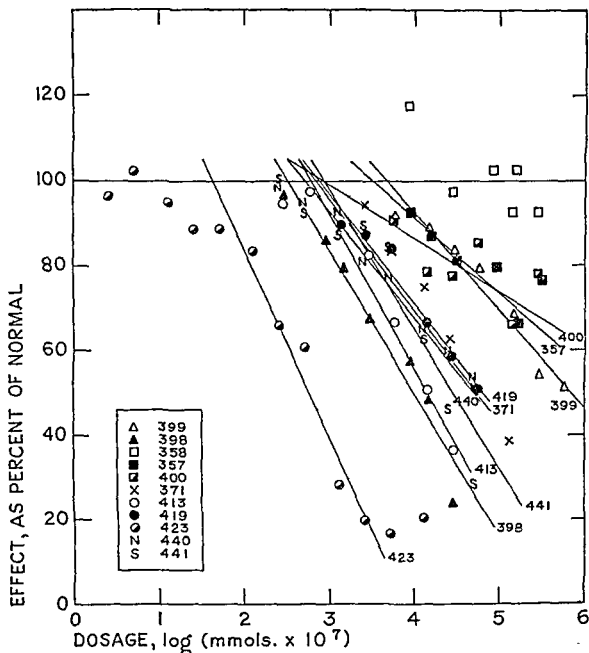


FIG. 3. GRAPH OF DOSE:RATE

The mean effects of the drugs upon the respiratory rate of rabbits breathing air have been plotted against dosage. The lines that have been drawn to fit the purely depressant effects correspond to equations given in table 3. A "threshold" dose is the dosage where a line intersects the axis of 100 per cent.

In figure 2, the utilization of oxygen after various doses of different drugs is seen to fall to 90, 80, or even (with amyldihydromorphinone, #423) about 70 per cent of normal. The points are too erratic to be fitted by straight lines, and too crowded to allow room in this figure for drawing smoothed curves.



Respiratory rate and minute volume (figs. 3 and 4) were influenced by the drugs in a more profound and regular way than metabolism, according to our measurements. The smallest doses injected left them unaffected or slightly diminished. As larger quantities were tried the respiration began

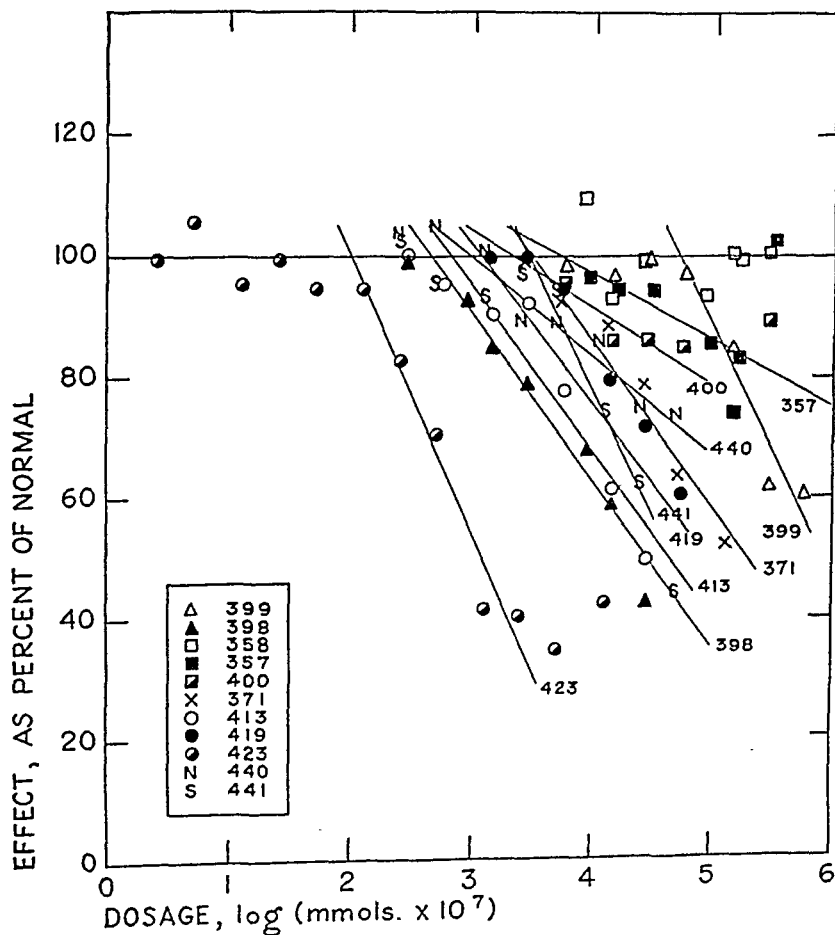


FIG. 4. GRAPH OF DOSE: MINUTE-VOLUME, LIKE THAT OF DOSE:RATE IN FIG. 3

to fall an approximately equal fraction for each increment in the logarithm of the dosage. The different drugs differed in the level of dosage at which this happened and in the steepness of the fall. Respiratory rate always descended faster than minute volume, reaching as low as 17 per cent of normal with amyldihydromorphinone (#423). The largest doses given of amyldi-



hydromorphenone (#423) and methyldihydrocodeinone (#357) caused an amount of restlessness in the rabbits which counteracted depression to some extent.

The effect of substituting the extra functional group in ring-III was disappointing but interesting in its inconsistency. This is apparent in table 4, in which "threshold" doses of the present set of drugs and their analogs are drawn up in two parallel columns. It is still more striking in table 5, a re-

TABLE 4  
"Threshold" doses for depressing respiratory minute volume

UNSUBSTITUTED DRUGS			SUBSTITUTED DRUGS		
Name	Mgm.	Dose log (mmoles. $\times 10^3$ )	Name	Mgm.	Dose log (mmoles. $\times 10^3$ )
#37. Dihydromorphine	0.11	3.6	#399. Methyldihydromorphine	1.54	4.7
123. Dihydromorphinone	0.01	2.6	398. Methyldihydromorphinone	0.01	2.6
123. Dihydromorphinone	0.01	2.6	413. Ethyldihydromorphinone	0.02	2.8
123. Dihydromorphinone	0.01	2.6	419. Isopropylidihydromorphinone	0.10	3.5
123. Dihydromorphinone	0.01	2.6	423. Amyldihydromorphinone	0.004	2.0
123. Dihydromorphinone	0.01	2.6	440. Benzylidihydromorphinone	0.04	3.0
123. Dihydromorphinone	0.01	2.6	441. Phenylidihydromorphinone	0.10	3.5
20. Dihydrocodeine	0.92	2.5	358. Methyldihydrocodeine	*	*
154. Dihydrocodeinone	0.08	3.4	357. Methyldihydrocodeinone	0.18	3.8
371. Dihydrocodeinone enol acetate	0.11	3.5	400. Methyldihydrocodeinone enol acetate	0.07	3.3

\* No dose tried (0.3-10 mg.) was depressant.

arrangement of the information in table 4, to make horizontal distances represent the differences among the drugs. The more depressant a drug is, the further to the left in table 5 its symbol stands. In seven pairs of compounds out of ten, the substituted one was less depressant. Methyldihydrocodeine (#358), deprived entirely of ability to depress, was the extreme case. The aromatic radicals (in benzyl-, #440, and phenyl-dihydromorphinone, #441) made about as much difference as some of the alkyl. The weight of the alkyl groups seems to have been unimportant: through the first three members



of the series, methyl, ethyl, isopropyl (in #398, #413, #419), potency dwindled with increasing weight, but then amyl (in #423) formed the most powerful drug of all derivatives of morphine whose respiratory effects have been studied in this way. Whether substitution is performed in a morphine, a morphinone, a codeine, or a codeinone seemed to make no measurable difference. One can only predict, on the basis of these results, that the substitution of an extra group in ring-III of morphine or its derivatives is apt to weaken a drug as a depressant for the respiration of rabbits; although not by a constant decrement, and not invariably.

TABLE 5  
*"Threshold" doses as influenced by substitution\* in ring-III*

DRUGS	"THRESHOLD" DOSE AS LOG (MMOLES. $\times 10^7$ )																
	2.0	2.1	2.2	2.3	2.4	2.5	2.6	2.7	2.8	2.9	3.0	3.1	3.2	3.3	3.4	3.5	3.6
#399 vs. 37																	U
398 123							US										
413 123							U	S								S	
419 123							U										
423 123	S						U										
440 123							U			S							
441 123							U								S		
358 20																	U
357 154														S			
400 371											S		U				
																S	Higher than any dose tried

\* S = substituted, U = unsubstituted.

Since substitution has such variable effects, one looks for an explanation in the individualities of the various substituents, but unsuccessfully. On the one hand, like effects resulted from unlike substituents: the difference due to isopropyl (in isopropylidihydromorphinone, #419) was like that due to phenyl (in phenylidihydromorphinone, #441); the difference due to benzyl (in benzylidihydromorphinone, #440) was like that due in one instance to methyl (in methylidihydrocodeinone, #357). It is not hard to imagine ways in which two different radicals might happen to cause nearly the same effects; but it is hard to explain why, on the other hand, the one group, methyl, caused such diverse effects. It weakened depressant power in three instances (methylidihydromorphine, #399, methylidihydrocodeine, #358, methylidihydrocodeinone, #440).



hydrocodeinone, #357), strengthened it in one (methyldihydrocodeinone enol acetate, #400), and made no difference in one (methyldihydromorphinone, #398). Hence it can not be said that the nature of the radical is the all-important determinant of results.

The chemists, when pressed by the pharmacologists in this difficulty, admit that the procedures used in substituting the methyl radical in ring-III could at times, conceivably, have changed some other portion of the molecule as well, but they consider it unlikely. Preferable as an explanation is the possibility that radical and molecule affect one another's influence in a way too complex to be represented by simple algebraic summation. A methyl group attached to dihydromorphine may be a very different thing from a methyl group attached to dihydromorphinone, to dihydrocodeine, to dihydrocodeinone, or to dihydrocodeinone enol acetate.

#### SUMMARY

Ten drugs differing from morphine and related compounds by the attachment of an additional radical to ring-III in the molecule have been given in various dosages to rabbits to see how this chemical change affected the power to depress respiration. They are methyldihydromorphine, methyldihydromorphinone, methyldihydrocodeine, methyldihydrocodeinone, methyldihydrocodeinone enol acetate, ethyl-, isopropyl-, amyl-, benzyl-, and phenyl-dihydromorphinone. An eleventh substance, dihydrocodeinone enol acetate, was also studied to complete our background of analogous unsubstituted compounds. Judged by the "threshold" dose to depress respiratory minute volume, the new type of chemical alteration was found to be correlated with increased potency in two instances, with diminished potency in seven, and with no change in one. Amyldihydromorphinone was more potent than any other of the sixty-five derivatives of morphine whose respiratory effects have been studied in this laboratory. The varying result of substitution can not be accounted for entirely by the individuality of the extra radical, since one case of augmented strength, three cases of reduced strength, and the case of no change, all followed substitution of the methyl group. No satisfactory explanation is known.

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# THE RESPIRATORY EFFECTS OF MORPHINE, CODEINE, AND RELATED SUBSTANCES

## XI. THE INFLUENCE OF THE ACETOXYL GROUP<sup>1</sup>

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Received for publication April 19, 1941

How the acetoxyl group, in certain derivatives of morphine, affects their power to depress the respiration of rabbits has already received some attention in one publication from this laboratory (1). In the present paper we will describe the respiratory effects of 5 more acetylated compounds; and will take the occasion to review for substances derived from morphine the influence upon respiration of acetoxyl compared with other groups.

### THE DRUGS

The drugs were prepared in the laboratory of Lyndon F. Small at the University of Virginia, and were sent to us with descriptive data concerning their physical properties which are summarized in table 1. In all of them an acetoxyl group has replaced the secondary alcoholic hydroxyl group present in morphine at carbon-6. Only one of them, however, is a morphine. In the others, additional chemical changes have produced a codeine, an isocodeine, and the hydrogenated forms of these. Figure 1 shows their structural formulas.

### EXPERIMENTS AND RESULTS

The experiments were conducted in the routine way that has been used in this series of studies (2, 3). Immediately before the subcutaneous injection of drug, and again one hour later, we measured the consumption of oxygen, respiratory rate, and respiratory minute volume of rabbits breathing room air; the respiratory rate and minute volume, breathing about 8 per cent CO<sub>2</sub>; and the heart rate. Each observation after an administration of

<sup>1</sup> The work reported in this paper is part of a unified effort by a number of groups to solve the problem of drug addiction. The participating organizations have been the Rockefeller Foundation, the National Research Council, the United States Public Health Service, the United States Bureau of Narcotics, the Massachusetts Department of Health, the University of Virginia, and the University of Michigan.



drug was divided by the corresponding figure for the same individual before injection. Expressed thus, as percentages, the results were averaged.

For presenting the average results the plan followed and discussed in an earlier paper (3) will be used. The results are all assembled in table 2. Those concerned with oxygen consumption, respiratory rate, and respiratory minute volume, while the animals were inhaling air, have been plotted against dosage in figures 2, 3, and 4. Those describing respiratory rate and minute

TABLE 1  
*Physical properties of the drugs*

DRUG	MOLECULAR WEIGHT	BASIC FRACTION	MELTING POINT	APPROXIMATE SOLUBILITY PARTS PER 100 OF H <sub>2</sub> O	TEMPERATURE OF SOLUBILITY TEST	OPTICAL ROTATION		
						[α] <sub>D</sub>	Temperature	Concn.
		per cent	°C.		°C.		°C.	
375.† Monoacetyl-α-isomorphine hydrochloride	363.6	90.0	241-242*	5	29	-223	30	1.34 water
366. Acetylcodeine hydrochloride	377.5	90.3	180-201	very				
367. Acetyldihydrocodeine hydrochloride	379.7	90.5	235	20	room temp.	-101.2	27	1.126 water
318.† Acetylisocodeine hydrochloride (+ 4.5 H <sub>2</sub> O)	458.7	74.4	107-109	50	cold water	-216	27	0.42 water
390.† Acetyldihydroisocodeine acid tartrate	511.3	67.2	196-198	25	room temp.	-58	27	0.75 water

\* *In vacuo.*

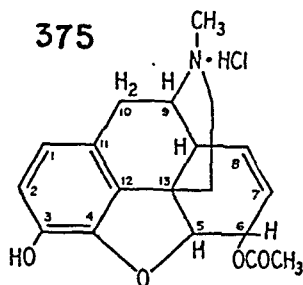
† New drug.

volume, both on air and on the CO<sub>2</sub> mixture, at different dosages, were fitted by the method of least squares with straight lines, whose equations are given in table 3. (Data used in calculating these equations are indicated in table 2 by asterisks.) The lines for the condition of rebreathing room air, appear in the figures, along with the plotted points. Each predicts a "threshold" dose, *T*. The value of *T* for minute volume on air is the criterion by which the potency of the drugs will be judged.

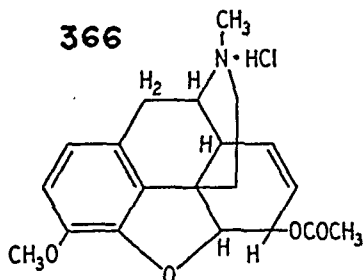
Although a large amount of information is provided by the tables and graphs, the discussion of results will be confined to a few summarizing re-



375

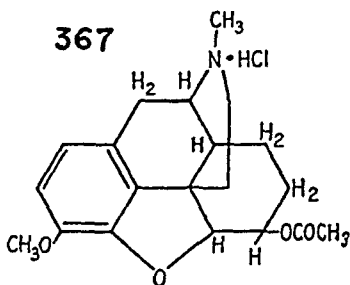
Acetyl  $\alpha$  isomorphine

366



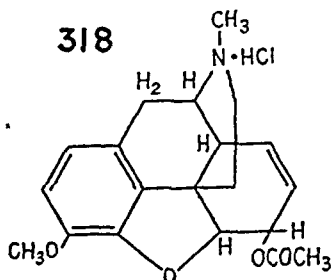
Acetylcodeine

367



Acetyldihydrocodeine

318

Acetyl-  
isocodeine

390

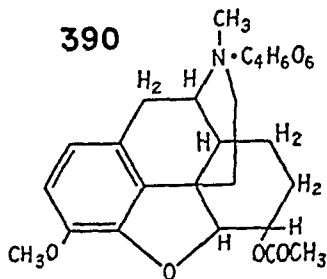
Acetyldihydro-  
isocodeine

FIG. 1. THE STRUCTURAL FORMULAS OF THE DRUGS



marks about the effects of the 5 substances presented here, and a comparison of their depressant power with that of analogous compounds.

#### DISCUSSION

This is an active set of drugs. One of them, monoacetyl- $\alpha$ -isomorphine (#375), halved the heart rate, while the other four reduced it to four-fifths of its original value (see table 2).

As usual with derivatives of morphine, the consumption of oxygen was not greatly affected. The trend of the average utilization after small doses of several of the drugs suggests (see fig. 2) that quantities too small to depress were slightly stimulating, but the values of the standard deviations in table 2 show that no one of these above-normal averages differs from normal by an amount that is significant. Depression once having set in, 77 per cent of normal, with acetyl- $\alpha$ -isomorphine (#375), was the lowest average value that was observed. The two unhydrogenated codeines depressed to about 85 per cent. This is a larger reduction of metabolism than that caused by morphine in quantities equally depressant for rate and minute volume. The two hydrogenated codeines depressed hardly at all.

For all of the compounds the rate of breathing and the minute volume fell increasingly with increasing doses, as figures 3 and 4 show. Monoacetyl- $\alpha$ -isomorphine (#375) again was the strongest of the 5. Other features of figures 3 and 4 are alike. Both show that the greatest reduction resulted not from the largest dose given but from something less. As the quantity of drug increased beyond that point, the respiration began to return toward normal. (Although acetylisocodeine (#318) is an exception, restlessness which developed in the rabbits after the largest dose of it seemed to suggest that with further increase of dose it would probably have done as the other drugs did.) Both graphs show an interesting pattern among the four codeines. They tend to pair two ways, acetylcodeine (#366) and acetyldihydrocodeine (#367) having slopes much alike and much shallower than their two isomers', while acetyldihydrocodeine (#367) and acetyldihydroisocodeine (#390) have practically identical threshold doses, that are considerably larger than those of their unsaturated analogs. In other words, a change in spatial configuration seems to affect the rate at which depression increases with dosage, whereas the presence of the double bond (carbon 7-8) seems to affect threshold potency. We know of no good explanation of any of these relationships.

So much can be gleaned from comparisons within the present set of 5 drugs. To multiply the comparisons, we have placed in table 4 all the acetoxy derivatives of morphine whose effects upon the respiration of rabbits have been studied in the drug addiction laboratory (1, 2, 4). Aligned with them are all the analogous compounds for which similar data are available (2, 5-10). The gaps in the table represent compounds which either have



TABLE 2  
Mean drug effect† upon heart rate and respiratory functions

DOSAGE		NUMBER OF ANIMALS	MEAN EFFECT											
Mgm.	Log (minoles. X 10 <sup>4</sup> )		Heart rate		Oxygen consumption		Rate				Minute volume			
			Per cent	$\sigma$	Per cent	$\sigma$	Air		CO <sub>2</sub>		Air		CO <sub>2</sub>	
			Per cent	$\sigma$	Per cent	$\sigma$	Per cent	$\sigma$	Per cent	$\sigma$	Per cent	$\sigma$	Per cent	$\sigma$
Controls														
Drug #375. Monoacetyl- $\alpha$ -isomorphine hydrochloride														
0.0		39	100.9	7.8	102.1	9.5	101.5	21.1	101.9	9.3	99.1	14.5	101.7	10.9
0.00047	.111	2	100.0a		0.0	0.0	92.0	24.0	106.5	7.7	112.5	4.9	121.0	4.2
0.005	1.138	7	108.0	16.2	99.3	13.0	98.7	16.9	101.6	16.5	97.3	10.7	107.0	17.8
0.01	1.439	8	97.8	10.7	102.5	6.4	94.6	19.9	102.5	15.1	98.8	8.3	106.3	8.9
0.02	1.740	8	98.6	12.3	104.4	12.6	92.0*	4.6	93.3*	11.1	96.6*	10.3	95.5*	13.6
0.05	2.138	13	96.8	7.0	98.8	8.7	79.7*	15.2	85.4*	9.4	86.5*	12.8	83.0*	10.6
0.1	2.439	8	93.3	8.1	91.5	13.0	64.5*	18.8	73.5*	12.8	75.3*	16.3	71.4*	19.4
0.2	2.740	12	87.5	14.6	83.6	15.6	51.8*	13.2	53.8*	12.2	63.8*	14.9	50.8*	18.6
0.5	3.138	7	64.7	12.2	77.7	14.7	31.3*	10.4	33.0*	14.8	37.6*	9.2	27.1*	9.6
1.0	3.439	7	56.1	20.6	80.0	19.6	33.0*	19.5	25.7*	10.2	43.3	19.6	23.4	8.9
2.0	3.740	4	52.8	14.2	91.3	6.1	34.0	7.3	28.0	16.8	48.8	8.4	23.0	7.9
a: n = 1														
Drug #366. Acetylcodeine hydrochloride														
0.05	2.122	8	96.0	8.2	101.5	9.5	94.1*	7.2	91.4*	8.1	96.0*	12.0	87.8*	10.5
0.1	2.423	8	99.0	10.7	102.3a	5.4	83.8*	12.4	87.1*	5.1	94.3*	8.1	85.4*	10.3
0.2	2.724	8	94.5	4.2	97.5	11.3	82.9*	13.4	89.9*	9.0	87.5*	12.3	85.0*	13.0
0.5	3.122	7	94.1	8.3	106.0	13.3	82.1*	12.0	81.6*	13.6	92.0*	6.9	75.9*	16.2
1.0	3.423	8	94.3	4.0	102.1	12.1	73.4*	8.4	71.9*	6.9	80.5*	8.3	67.1*	9.8
2.0	3.724	8	89.0	9.2	91.3	14.1	70.5*	9.1	69.8*	5.4	81.1*	7.9	66.0*	5.4
5.0	4.122	7	91.0	7.3	84.6	10.6	49.8*	17.1	60.7b*	14.5	67.2*	13.8	54.2b*	30.5
10.0	4.423	8	84.8	16.4	96.4	15.2	63.4	19.0	59.4	10.4	70.8	10.1	46.8	9.4
a: n = 4      b: n = 6														

a: n = 1

b: n = 6

a: n = 4



Drug #367. Acetyldihydrocodeine hydrochloride

0.2	2.722	11	99.1	4.9	110.9	19.5	89.5	9.5	96.6	6.1	98.1	10.0	96.5	9.6
0.5	3.120	7	93.4	8.5	111.3	23.2	93.6*	16.1	96.6*	7.6	95.9*	14.1	93.0*	10.4
1.0	3.421	8	100.3	6.4	105.0	12.3	87.4*	12.3	93.3*	8.3	95.3*	13.0	91.4*	10.9
2.0	3.722	7	100.6	5.9	102.9	11.7	88.7*	11.6	87.7*	7.1	91.1*	11.0	86.7*	10.8
5.0	4.120	8	98.0	9.8	99.4	18.2	73.5*	12.9	74.3*	7.2	83.0*	11.0	70.4*	9.4
10.0	4.421	8	100.1	12.3	96.8	11.5	70.3*	20.9	74.5*	16.4	79.0*	16.0	67.0*	20.3
20.0	4.722	8	81.9	13.5	137.1	32.7	80.6	37.8	77.0	30.4	102.9	36.8	75.1	39.4

Drug #318. Acetylcodeine hydrochloride

0.1	2.338	8	99.8	7.9	109.1	9.0	93.3	10.2	97.3	7.7	96.0	9.7	95.0	9.1
0.2	2.640	8	103.4	6.5	100.1	10.7	90.0*	14.8	94.4*	7.0	92.4*	9.8	94.8*	8.5
0.5	3.037	8	99.0	7.9	101.6a	17.7	79.0*	11.2	85.9*	10.5	83.5*	12.9	83.0*	10.7
1.0	3.338	8	91.4	9.4	87.6	14.8	58.4*	16.9	74.6a*	13.8	71.8*	15.7	73.4a*	16.0
2.0	3.640	8	95.5	8.5	91.5	4.7	64.4*	14.0	66.4*	7.5	78.0*	12.3	64.8*	9.3
5.0	4.037	7	80.1	13.0	86.1	18.8	43.7*	9.7	51.7*	10.3	55.0*	9.9	43.1	3.0
10.0	4.338	7	77.5	21.5	90.0b	13.4	31.8b*	19.3	37.1b*	20.9	50.4b*	21.2	34.0b*	20.8

a: n = 7    b: n = 8

Drug #390. Acetyldihydroisocodeine acid tartrate

0.2	2.592	8	98.5	6.3	99.8	8.4	101.5	6.1	99.1	10.5	102.6	7.7	100.4	9.7
0.5	2.990	7	102.9	3.8	105.7	12.5	88.1*	12.6	91.4*	4.7	98.3*	10.1	92.4*	7.8
1.0	3.291	8	95.6	7.6	102.5	7.4	77.1*	10.6	84.8*	5.8	89.6*	7.6	81.6*	6.1
2.0	3.592	8	91.3	4.9	96.1	15.4	66.9*	10.3	66.6*	9.4	75.9*	8.9	62.4*	9.6
5.0	3.990	8	71.6	17.1	99.1	13.7	35.9*	19.8	34.6*	16.5	50.3*	10.6	30.8*	13.7
10.0	4.291	7	63.9	20.1	96.4	19.1	40.4	8.2	34.3	14.2	58.0	14.2	30.7	14.2

\* Effects used in calculating the lines of figures 3 and 4 and table 3.

† The data from columns 2 and 6 are plotted in figure 2; from columns 2 and 8, in figure 3; from columns 2 and 12, in figure 4.



TABLE 3  
*Linear equations\* to describe dosage-effect relationships*

RESPIRATORY FUNCTION	CONSTANTS							
	m	b		T				$\sigma$
		For dose in mgm.	For dose in mmoles. $\times 10^6$	Mgm.	Log mgm.	mmoles. $\times 10^6$	Log (mmoles. $\times 10^6$ )	
Drug #375. Monoacetyl- $\alpha$ -isomorphine hydrochloride								
Rate air.....	39.0	24.5	160.5	0.012	-1.935	36	1.550	14.3
Rate CO <sub>2</sub> .....	44.0	22.4	175.8	0.017	-1.763	53	1.722	11.7
M.V. air.....	40.9	29.9	172.4	0.019	-1.715	59	1.770	12.9
M.V. CO <sub>2</sub> .....	49.9	14.0	187.3	0.019	-1.732	57	1.753	13.4
Drug #366. Acetylcodeine hydrochloride								
Rate air.....	17.5	70.4	131.0	0.020	-1.694	59	1.773	11.6
Rate CO <sub>2</sub> .....	15.5	73.2	126.8	0.018	-1.735	54	1.733	8.7
M.V. air.....	12.4	81.0	124.0	0.029	-1.531	86	1.936	10.2
M.V. CO <sub>2</sub> .....	16.8	68.3	126.5	0.013	-1.890	38	1.577	11.6
Drug #367. Acetyldihydrocodeine hydrochloride								
Rate air.....	18.6	88.1	152.5	0.23	-0.641	660	2.823	13.9
Rate CO <sub>2</sub> .....	19.4	91.0	158.1	0.34	-0.467	1000	2.998	9.7
M.V. air.....	14.2	93.1	142.2	0.32	-0.491	940	2.974	11.3
M.V. CO <sub>2</sub> .....	22.6	88.4	166.6	0.31	-0.515	890	2.949	12.6
Drug #318. Acetylisocodeine hydrochloride (hydrated)								
Rate air.....	33.2	62.5	177.6	0.074	-1.129	220	2.337	14.6
Rate CO <sub>2</sub> .....	33.4	69.7	185.5	0.123	-0.908	360	2.559	11.5
M.V. air.....	25.7	73.7	162.9	0.095	-1.021	280	2.446	14.3
M.V. CO <sub>2</sub> .....	36.5	67.0	193.4	0.124	-0.907	360	2.560	11.7
Drug #390. Acetyldihydroisocodeine acid tartrate								
Rate air.....	51.7	66.4	245.5	0.22	-0.650	650	2.815	13.6
Rate CO <sub>2</sub> .....	58.4	69.5	271.9	0.30	-0.522	880	2.943	11.5
M.V. air.....	48.6	78.5	247.9	0.36	-0.442	1050	3.023	10.0
M.V. CO <sub>2</sub> .....	62.8	66.9	284.5	0.30	-0.527	870	2.937	10.8

\* They were calculated from data given in table 2. The straight lines corresponding to these equations are drawn in figures 3 and 4. The equations have the form,  $Y' = -mX + b$ , where

$Y'$  = predicted effect, as per cent of the normal, at any given dose

$X$  = log dose in mgm. of base, or mmoles., per kilogram

$m$  = slope of the line, representing change in respiration (expressed as a per cent) for each unit of increase in log dose

$b$  = calculated effect, as per cent of normal, when log dose is 0. It has no pharmacological meaning when its value is more than 100 per cent.

M.V. stands for minute volume, T for "threshold" dose (dose for predicted effect of 100 per cent),  $\sigma$  for the standard deviation of the equation.



never been prepared, or have never been studied. The 5 names differentiated by asterisks are the substances whose effects have been newly described in this paper. For a criterion of potency each substance is accompanied by

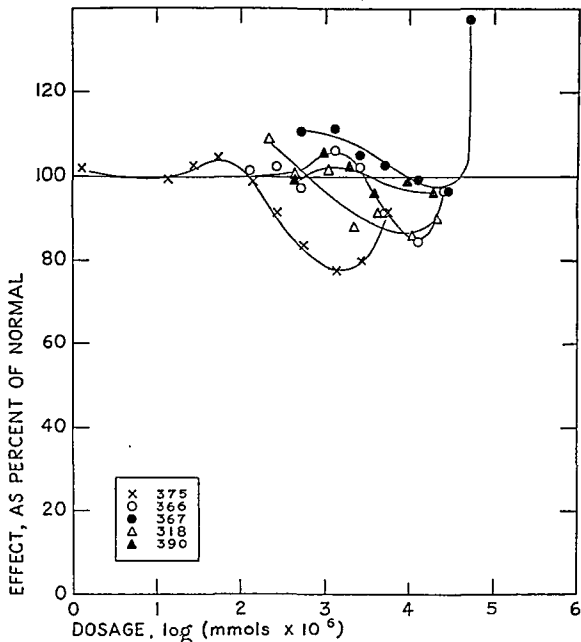


FIG. 2. THE MEAN EFFECTS OF THE DRUGS IN VARIOUS DOSES UPON CONSUMPTION OF OXYGEN

The smoothed curves were drawn by eye

a number which is its "threshold" dose for depressing minute volume when a rabbit breathes air. (Those attached to the present set of 5 drugs were taken from table 3.) Table 4 is useful to emphasize the chemical relationships among the compounds.

More convenient to show their pharmacological relationships is a different



arrangement, given in table 5, of the same information. Here drugs are represented by symbols which refer to their substituent groups. The symbols have been entered in columns appropriate for the corresponding "thresh-

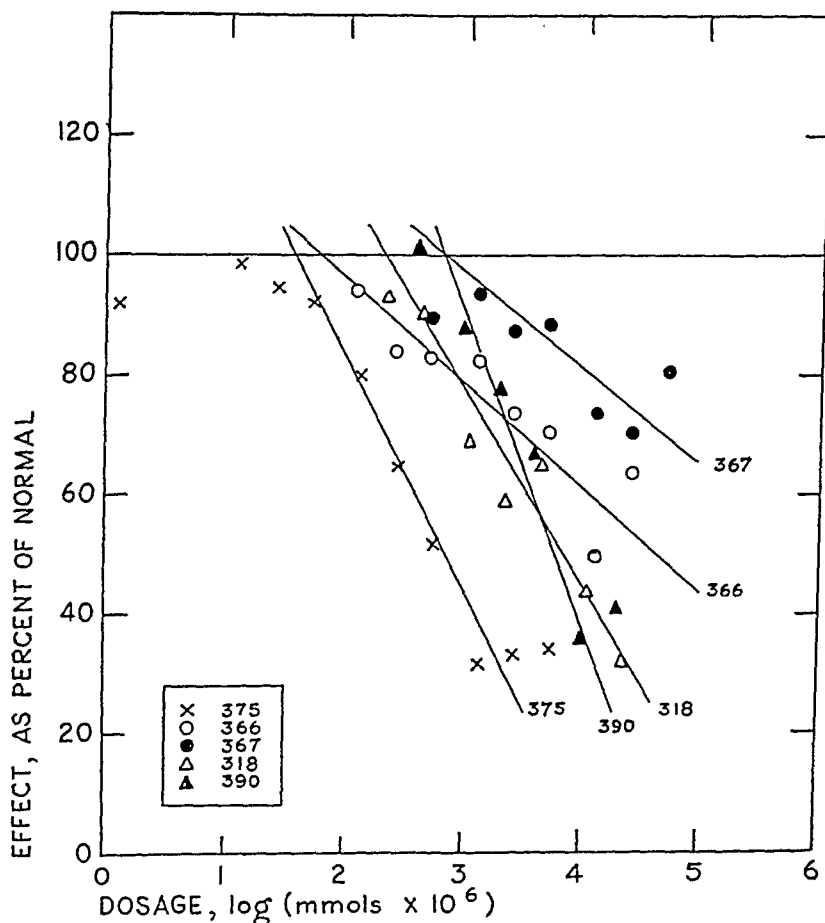


FIG. 3. GRAPH OF DOSE: RATE

Mean effects upon the respiratory rate of rabbits breathing air, as given in table 2, have been plotted against dosage. The straight lines that have been drawn to fit the purely depressant effects correspond to equations given in table 3. A "threshold" dose is the dosage where a line intersects the axis of 100 per cent.

old" doses to depress minute volume. The more depressant a drug is, the farther to the left it stands in this chart. The horizontal rows correspond by number to the rows in table 4.



In this assembly of compounds are 14 pairs in which the acetoxyl group can be compared to the hydroxyl in influence upon power to depress the rabbit's respiration. Acetoxyl confers the greater potency, 12 out of 14 times. Among the 10 pairs in which acetoxyl can be compared to methoxyl,

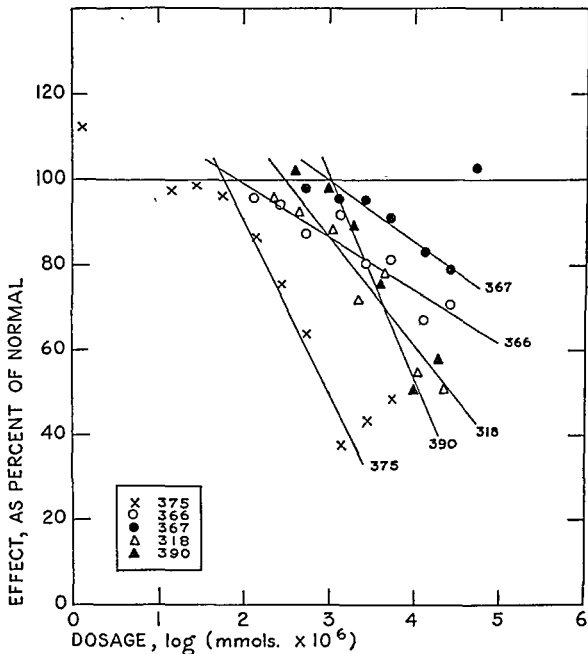


FIG. 4. GRAPH OF DOSE:MINUTE-VOLUME, LIKE THAT OF DOSE:RATE IN FIG. 3

acetoxyl is superior in 6 instances. In comparison to the ethoxyl group or the hydrogen atom, on the other hand, the acetoxyl group proves always inferior. The number of pairs available to substantiate the last statement, however, is relatively small. One finds, while comparing acetoxyl with the other substituents, that the replacement of two methoxyl radicals or two



TABLE 4  
 "Threshold" doses† of acetoxy, and parallel, derivatives of morphine

	AcO		EtO		MeO		OH		H
	Acetoxy on C-6		Ethoxy on C-6		Methoxy on C-6		Hydroxy on C-6		Hydrogen on C-6
1	6-Acetylmor- 1.76 phine #199		Morphine-6- 1.54 ethyl ether #296		Morphine-6- 1.73 methyl ether #111		Morphine #1 2.72		
2	6-Acetyldihy- 2.34 dromorphine #129		Dihydromor- 1.54 phine 6-eth- yl ether #297		Dihydromor- 1.90 phine 6- methyl e- ther #112		Dihydromor- 2.63 phine #37		Dihydrodes- 1.64 oxymor- phine-D #126
3	*6-Acetyl- $\alpha$ - 1.77 isomorphine #375				$\alpha$ -Isomor- 1.52 phine 6- methyl e- ther #302		$\alpha$ -Isomor- 2.72 phine #264		
4	*Acetylcodeine 1.93 #366				Codeine 3.20 methyl e- ther #85		Codeine #2 3.64		
5	*Acetyldihy- 2.97 drocodeine #367				Dihydrocode- 2.80 ine methyl ether #87		Dihydrocode- 3.43 ine #20		Dihydrodes- 2.45 oxycodine- D #16
6	*Acetylisocod- 2.45 ine #318						Isocodeine 3.70 #36		
7	*Acetyldihy- 3.02 droisocode- ine #390						Dihydroiso- 2.66 codeine #61		Dihydrodes- 2.45 oxycodine- D #16
8	Dihydrocode- 2.50 inone enol acetate #371				Dihydrotho- 3.20 baine #97				
9	Methyldihy- 2.32 drocodeinone enol acetate #400								
	Acetoxy on C-3		Ethoxy on C-3		Methoxy on C-3		Hydroxy on C-3		Hydrogen on C-3
10	Diacetylmor- 1.61 phine #127				*Acetylcode- 1.93 ine #365		6-Acetylmor- 1.76 phine #199		
11	Diacetyldihy- 2.53 dromorphine #128				*Acetyldihy- 2.97 drocodeine #367		6-Acetyldihy- 2.34 dromor- phine #129		
	Acetoxy on C-14		Ethoxy on C-14		Methoxy on C-14		Hydroxy on C-14		Hydrogen on C-14
12	Acetylhydroxy 2.83 codeinone #321						Hydroxycod- inone #320, no depres- sion		



TABLE 4—Continued

	AcO	EtO	MeO	OH	H
	Acetoxyl on C-3 and C-6	Ethoxyl on C-3 and C-6	Methoxyl on C-3 and C-6	Hydroxyl on C-3 and C-6	Hydrogen on C-3 and C-6
13	Diacetylmor- 1.61 phine #127		Codeine meth- 3.20 yl ether #85	Morphine #1 2.72	
14	Diacetyldihy- 2.53 dromorphine #128		Dihydrocode- 2.80 ine methyl ether #87	Dihydromor- 2.58 phine #37	
	Acetoxyl on C-6 and C-14	Ethoxyl on C-6 and C-14	Hydroxyl on C-6 and C-14	Hydroxyl on C-6 and C-14	Hydrogen on C-6 and C-14
15	Diacetyldihy- 2.72 drohydroxy- codeine-B #391			Dihydroby- 3.37 droxycode- ine-C #370	
16	Diacetyldihy- 2.43 drohydroxy- codeine-C #392			Dihydroby- 3.07 droxycode- ine-B #324	

\* A drug whose respiratory effects are first described in this paper.

† Doses are log (m moles  $\times 10^4$ ) per kilogram.

hydroxyls with two acetoxyls (rows 13, 14, 15, and 16) gives increased potency just as single replacement, at carbon-6, does.

To explain the differences, we do not know of any supplementary facts adequate even as clues. The order of depressant potency among the compounds does not duplicate the order of any other single property among them, such as solubility, molecular weight, or ease of hydrolysis. Rizzotti (11) has reported that a small quantity of diacetylmorphine in 5 hours of perfusion through the isolated heart of the frog changed completely to 6-acetylmorphine; the latter after further contact with the beating heart changed, in part, to morphine. Wright (12, 13) has found the serum of rabbits to catalyze the deacetylation of some acetylated derivatives of morphine. Results such as these emphasize that the relative potency of these drugs depends not only upon their immediate physical and chemical properties but also upon those of the products into which they transform, and hence upon the relative speeds of the transformations.

Table 5 brings out another fact: that the nature of the group substituted at carbon-6 affects potency less in hydrogenated compounds than in unhydrogenated ones. For example, in row 1, the "threshold" doses of morphine-6-methyl ether, 6-acetylmorphine, and morphine spread from 1.7 to 2.7; in row 2, the "threshold" doses of hydrogenated forms of the same drugs spread only from 1.9 to 2.6. Again, in row 4, the range for acetylcodeine, codeine methyl ether, and codeine is from 1.9 to 3.6; in row 5, for the hy-



A graphic presentation of the data in table 4

"THRESHOLD" DOSE AS LOG (mmoles  $\times 10^6$ )

Group varying on C-6																	Higher than any dose given								
	1	1.5	1.6	1.7	1.8	1.9	2.0	2.1	2.2	2.3	2.4	2.5	2.6	2.7	2.8	2.9		3.0	3.1	3.2	3.3	3.4	3.5	3.6	3.7
Group varying on C-3	1	EtO		MeO	AcO																				
	2	EtO	H			MeO				AcO															
	3	MeO			*AcO										OH					MeO			OH		
	4					*AcO																			
	5															MeO		*AcO				OH			OH
	6												H												
	7											*AcO													OH
	8												H												
Group varying on C-3	9									AcO										MeO					
	10		AcO																						
	11																								
Group varying on C-14	12																								OH
	13																								
Group varying on C-3 and C-6	14																								
	15																								
Group varying on C-6 and C-14	16																								

\* A drug whose respiratory effects are first described in this paper.

† AcO = acetoxy, EtO = ethoxy, MeO = methoxy, OH = hydroxy, H = hydrogen.



drogenated forms, from 2.8 to 3.5. - The same observation can be repeated with double substitutions: in row 13, the range is 1.6 units, whereas in row 14, it is 0.3 units.

The reason for this difference also is entirely obscure. Numerous comparisons (10, pp. 26-29) between hydrogenated and unhydrogenated derivatives of morphine have failed to discover any generalization about the pharmacological significance of hydrogenation among them. If attention is restricted, however, to only the acetoxyl and hydroxyl groups in table 5, the effect of hydrogenation is reminiscent of Wright's finding (13) that an acetoxyl group attached either to carbon-3 or to carbon-6 in an unhydrogenated morphine nucleus is hydrolyzed more readily by rabbit serum than when attached in a dihydromorphine nucleus. We are tempted into speculating on a possible connection between these observations as follows:

1. The 6-acetoxyl group has been found by the experiments here reported to affect potency. Hence it must share in the mechanism by which the drug produces its effect.

2. The 6-acetoxyl group might act *a)* while attached to the nucleus, or *b)* after detachment from the nucleus and breakdown or attachment to something else, or *c)* in the act of being detached.

3. The acetoxyl group is so common in normal physiological economy, that a small additional concentration due to that released from a drug is not likely to exert a specific effect. Thus, the *b)* possibility need not be considered seriously.

4. If the 6-acetoxyl group affects potency while attached to the nucleus, either by affecting a physical property of the drug such as solubility, or a chemical feature such as combining power with tissue, its greatest effect on potency should appear among drugs to which it remains attached longest, i.e., in hydrogenated compounds.

5. If the 6-acetoxyl group affects potency in the process of its own removal, its greatest effect on potency should appear among the compounds most readily stripped, i.e., the unhydrogenated compounds.

6. Table 5 shows that the widest inter-drug differences appear among the unhydrogenated compounds.

7. Hence, if this reasoning holds, any effect contributed to the action of the molecule by the 6-acetoxyl group is exerted *c)* in the act of being detached.

#### SUMMARY

How the metabolism, respiratory activity, and heart rate of rabbits are affected by 5 acetylated drugs related to morphine has been studied. 6-Acetyl- $\alpha$ -isomorphine is more depressant than the 4 codeines, acetyl-, acetyldihydro-, acetyliso-, and acetyldihydro-isocodeine. Among the latter, hydrogenation is correlated with the higher "threshold" doses, and the



spatial position of the acetoxyl group designated by "iso-" is correlated with the more rapid decrease of respiratory activity as dosage increases.

Among 32 derivatives of morphine and codeine, the influence of the acetoxyl group upon the "threshold" dose for depressing the minute volume of rabbits was compared with that of ethoxyl, methoxyl, hydroxyl, or hydrogen. In 12 out of 14 pairs the acetoxyl compound has proved more strongly depressant than the one containing hydroxyl. Acetoxyl is more potent than methoxyl 6 out of 10 times. It is less potent, however, than ethoxyl or hydrogen in any of the comparable substances available. The hydrogenation of compounds containing acetoxyl, methoxyl, and hydroxyl, tends to obliterate differences in strength among them. Since hydrogenation makes it more difficult to hydrolyze acetoxyl compounds of morphine, we are led to guess that an acetoxyl group attached at carbon-6 exerts its characteristic influence while being detached, rather than as a part of the molecule.

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# CHANGES IN SUSCEPTIBILITY TO THE CONVULSANT ACTION OF METRAZOL<sup>1</sup>

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Received for publication June 25, 1941

It is well known that tolerance develops fairly rapidly to the convulsant action of metrazol under the conditions in which it is used in the treatment of schizophrenia. This was reported by von Meduna (4) in his paper introducing this form of therapy, and is commented on by most writers on the subject. The finding of Himwich and his collaborators (2) that these convulsions involve a marked degree of cerebral anoxia, indicates that cortical damage may be produced. Whitehead *et al.* (5) have indeed found areas of parenchymatous necrosis in the cerebral cortex in dogs and rabbits which had been subjected to a series of metrazol convulsions under the conditions encountered in the clinical use of the drug. It is conceivable that such partial decortication might lead to a tendency toward exaggerated motor responses to metrazol, but that this tendency is masked by the tolerance which develops. If such a latent increase in susceptibility were produced, it might become apparent when the tolerance is lost. Furthermore, if such an increased susceptibility is the result of the cortical damage resulting from the anoxia, it might be possible to demonstrate such a phenomenon directly, by inducing the series of convulsions under conditions in which tolerance does not develop.

Implicit in the foregoing is the assumption that the mechanism of tolerance is different from that which is responsible for the increased susceptibility. If this is the case, it should be possible to produce tolerance in an animal which has previously been rendered susceptible, by the same procedure which gives rise to tolerance in the normal animal. The experiments reported here were undertaken to study these various possibilities, using the white rat as the experimental animal.

## EXPERIMENTAL PROCEDURES AND RESULTS

### *1. Development of latent susceptibility and tolerance*

For this, a procedure was used which would approximate as closely as possible the clinical use of metrazol. Injections were made on alternate

<sup>1</sup> This study is part of the program of "Studies of Abnormal Behavior in the Rat" directed by Norman R. F. Maier and supported by a grant from the John and Mary R. Markle Foundation.



days, using a 1 per cent solution of metrazol<sup>2</sup> in 0.85 per cent NaCl, by the intraperitoneal route. The initial dose was 30 mgm. per kilogram. This caused a convulsion in only one rat of the 10 used. In this one, the dose of 30 mgm. per kilogram was repeated on alternate days until it failed to elicit a convulsion on two consecutive occasions. Then 35 mgm. per kilogram was given, until it also failed to evoke a convulsion on two consecutive trials. Further increments of 5 mgm. per kilogram were then given as indicated. In the other 9 animals, the second dose used was 35 mgm. per kilogram, and this was increased as indicated. This was continued to six weeks. Some degree of tolerance developed in each case. The animals remained in good condition during the first part of the experimental period; many of them gained weight for the first three weeks. During the last 10 days they began to lose weight, and the injections were discontinued on this account.

A period of 4 weeks was then allowed, during which no metrazol was administered. It was considered that this time would be adequate for the loss of the tolerance that had developed. To determine whether an increase in susceptibility had been produced, each rat was given an injection of metrazol, in the amount of 10 mgm. per kilogram less than the dose which had been necessary to elicit the first convulsion. In seven cases this dose was now effective in producing a convulsive response. In these, injections were made at weekly intervals, in amounts decreased each time by 5 mgm. per kilogram, until a dose was found which failed to elicit a convulsion. In the remaining 3 a trial was made a week after the first injection with a dose lower by 5 mgm. per kilogram than the one which had evoked the initial convulsion. In 2 of these this dose was effective; only in one of the 10 animals was it necessary to give the initial convulsant dose to produce the response after this withdrawal period. The data on this experiment are shown in table 1. On the average, the convulsant dose was raised one-third, from 37.5 to 50 mgm. per kilogram, by injections given on alternate days. During the four week interval in which the drug was withheld the tolerance disappeared and was replaced by the susceptible state. The minimum convulsant dose was reduced to about one-half of that necessary at the height of tolerance, and to two-thirds of that which was effective initially.

## *2. Production of increased susceptibility without previous tolerance*

To demonstrate the development of increased susceptibility, an interval between injections of one week was chosen. It was felt that this was long enough so that the tendency to develop tolerance would be much less in evidence than when injections were made on alternate days. The dose initially given to this group was 25 mgm. per kilogram, increased by increments of 5 mgm. per kilogram until a convulsion was produced. When this happened the next dose given was 5 mgm. per kilogram less than the previously effective

<sup>2</sup> We wish to thank Dr. F. B. Western of the Bilhuber-Knoll Corp. for the supply of metrazol for these experiments.



one. If this now failed to cause a convulsion the dose was increased for the next injection. Depending upon whether or not a convulsion was evoked, the dose given the following week was decreased or increased by this increment of 5 mgm. per kilogram. In most cases the dose necessary to elicit the third convulsion was lower than the amount needed for the first one. After each animal had had three or four convulsions it was given a rest period of seven weeks. The injections were then resumed, the first dose being 5 or 10 mgm. per kilogram less than the minimum which had previously caused a convulsion. In many cases convulsions were evoked by these decreased doses. Progressively smaller doses were then given until the minimum convulsant dose for each animal was established. In a few instances the increased susceptibility did not become apparent until after the rest period. The data for

TABLE 1

*Development of tolerance and latent susceptibility to metrazol*

*Injections given on alternate days; dose increased by 5 mgm. per kilogram following two consecutive failures of a given dose to cause a convulsion. Four-week interval between two series of injections. All doses are given in mgm. per kilogram.*

RAT NUMBER	INITIAL CONVULSANT DOSE	MAXIMUM DOSE TO WHICH ANIMAL BECAME TOLERANT	MAXIMUM CONVULSANT DOSE GIVEN	MINIMUM CONVULSANT DOSE AFTER WITHDRAWAL PERIOD
1	50	55	60	20
2	35	45	50	30
3	35	45	50	25
4	45	55	60	35
5	40	55		40
6	30	40	45	20
7	35	40	45	25
8	35	40	45	30
9	35	45	50	25
10	35	40	45	25
Average...	37.5	46.0	50.0	26.5

this group are given in table 2. It is evident from this table that it was possible to induce a state in which a dose of metrazol significantly smaller than the initially effective one was able to cause convulsions. It is also evident that this increased susceptibility persisted through the period of seven weeks in which the drug was not administered. As a matter of fact the dose required to evoke a convulsion was smaller after the interval in 16 of the 26 animals, as compared with 4 in which larger amounts were needed. Also it was possible to reduce the minimum convulsant dose still further in most cases.

### *3. Development of tolerance in animals previously rendered susceptible*

Nine of the animals of the previous group were given a rest period of three months after the second series of injections, and then given a series of injec-



tions on alternate days, in the attempt to induce a state of tolerance. The dose initially used was, in each case, 5 mgm. per kilogram below the minimum one which had ever evoked a convulsion in that animal. In seven cases there

TABLE 2

*Development of increased susceptibility to metrazol injections at weekly intervals*

Injections at weekly intervals. Period of 7 weeks with no injection following third or fourth convulsion. Doses in mgm. per kilogram.

RAT NUMBER	INITIAL CONVULSANT DOSE	LOWEST EFFECTIVE DOSE BEFORE REST PERIOD	NUMBER OF CONVULSIONS REQUIRED TO MAKE THIS DOSE EFFECTIVE	INITIAL CONVULSANT DOSE AFTER REST PERIOD	MINIMUM CONVULSANT DOSE AFTER REST PERIOD
11	45	40	3	30	25
12	40	30	4	35	35
13	45	40	3	35	25
14	35	30	4	30	20
15	40	40	3	35	25
16	35	30	2	25	20
17	40	25	4	30	25
18	40	35	3	45	45
19	35	30	4	25	20
20	35	30	2	25	25
21	40	45	3	40	20
22	35	30	2	25	20
23	40	30	4	25	25
24	40	40	3	40	30
25	35	30	2	25	25
26	40	35	3	35	35
27	50	55	3	45	45
28	35	50	3	45	30
29	40	30	4	25	25
30	45	40	2	35	25
31	40	40	3	35	20
32	40	35	3	35	25
33	30	20	5	25	20
34	45	45	3	40	35
35	40	35	2	30	30
36	35	30	2	30	15
Average.....	39.2	35.4		32.7	26.6

was still evidence of increased susceptibility, as shown by the dose required to induce convulsion. Possibly the susceptibility was even greater than these figures indicate, for some tolerance may have developed during the early part



of the period of injections on alternate days. The dose was then increased in the same way as in the first group. In each case it was possible by this means to produce a state of tolerance, to the point at which the dose necessary to elicit a convulsion was raised, not only to the level which had been effective in the first instance, but well beyond this. In table 3, which gives the data for this group, it is seen that sufficient tolerance was produced so that doses above the initially effective ones, and far above the minimum ones later reached, could now be given without producing a convulsion.

TABLE 3

*Development of tolerance to metrazol in rats previously rendered susceptible*

Injections at weekly intervals until minimum convulsant dose established. Three months later, injections on alternate days begun; dose increased as tolerance developed. Doses in mgm. per kilogram.

INJECTIONS AT WEEKLY INTERVALS			INJECTIONS ON ALTERNATE DAYS	
Rat number	Initial convulsant dose	Minimum convulsant dose	Initial effective dose	Maximum dose to which tolerance developed, i.e., which failed to cause convulsion
11	45	25	35	60
12	40	35	40	40
13	45	25	30	50
14	35	20	30	35
24	35	25	35	45
27	35	25	20	45
28	35	30	30	45
29	45	25	35	50
30	40	20	30	35
Average....	39.4	25.6	31.7	45.0

## DISCUSSION

It is evident from these findings that it is possible to develop in the white rat an increased tolerance or an increased susceptibility to the convulsant action of metrazol. Apparently the two departures from the normal involve different mechanisms, since tolerance involves a state of latent increased susceptibility which becomes manifest when the tolerance is lost by withdrawal of the drug. Furthermore, the development of the increased susceptibility does not depend on the development of tolerance, and the tolerance can be developed even in the presence of the increased susceptibility. Quantitatively, the increase in susceptibility is apparently the same whether tolerance has been produced or not.

The convulsions and the increased susceptibility do not lead to any demonstrable alteration in the behavior of the animals. Direct observations were not made on the animals of these groups. However, another series of rats,



whose performance in a difficult discrimination problem had been established, was given a series of metrazol convulsions comparable to those represented in table 1. Repetition of the test situations after the convulsive experience showed that retention of the discrimination problem which had been previously learned and the manner of reacting to the test situation were not impaired (3).

As to the mechanisms involved in these processes, it seems evident that the increased susceptibility is the direct consequence of the convulsions. The cortical damage resulting from the anoxia during the convulsion might be the basis for such an effect. As has been indicated, if the inhibitory centers are affected more than the motor areas, an exaggerated response to a convulsion-producing situation would be expected. As for the increased tolerance resulting from injections on alternate days, one possibility is that the drug is more rapidly detoxified by the liver. The observations of Dille and Seeborg (1) indicate that the liver is the site of destruction of metrazol. The brief duration of action of the drug shows that this process of detoxication must be a rapid one, and it is not impossible that the process is even more rapid in the animal receiving frequent injections of the drug.

It is evident from the results that the tolerance which develops when the drug is given on alternate days is only temporary, whereas the increased susceptibility which develops from a series of convulsions is relatively permanent, in that it lasts for at least three months.

There is a significant point in these observations with regard to the clinical use of metrazol to produce convulsions. That is the possibility that the series of convulsions gives rise to a similar state of increased susceptibility to that produced in the experimental animal, and that such an increased susceptibility would become manifest if a second series of injections were to be undertaken after a lapse of some time following the conclusion of the first series.

#### SUMMARY AND CONCLUSIONS

1. Rats can be made tolerant to the convulsant action of metrazol by giving injections of adequate doses on alternate days.

2. Animals in which such a tolerance has been developed have a latent increased susceptibility produced by the convulsions. This latent susceptibility becomes manifest if the tolerance is lost by withdrawal of the drug.

3. A series of metrazol convulsions at such intervals that tolerance does not develop, leads to a state of increased susceptibility to the convulsant action of the drug.

4. This increased susceptibility is not completely lost over a period of several weeks or even months during which no injections of the drug are made.

5. Tolerance can be produced in such susceptible animals in the same way as it is produced in normal animals, by giving adequate doses on alternate days.



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# THE EFFECTS OF ESTRONE, DIETHYLSTILBESTROL AND TESTOSTERONE ON THE IN VITRO RESPONSE OF THE SPAYED RAT UTERUS TO PITOCIN

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Received for publication June 30, 1941

Many recent experiments have shown a definite relationship between the pattern of uterine motility at any given time and the elaboration of estrogenic hormones and progesterone by the ovary (1). It has been demonstrated that the uterus becomes increasingly inactive after ovariectomy and that its normal activities can be restored by ovarian transplants. Further work has shown that the administration of estrone to ovariectomized animals results in the return of motility the pattern of which is indistinguishable from that observed in the normal animal during estrus. Accompanying the loss of the normal uterine reactions following ovariectomy there is a decreased response to oxytocic drugs including pitocin.

In the present work we were concerned with the determination of the response to pitocin of the uteri of rats ovariectomized for varying periods up to 15 months. These responses were compared with those obtained from the uteri of rats ovariectomized for similar periods and treated with estrone, diethylstilbestrol, and testosterone respectively for a period of five days before the experiments. We were primarily concerned with the determination of the pattern of uterine motility under these different conditions and of the extent to which pre-treatment with the different hormones altered the response to a fixed dose of pitocin.

## METHODS

Animals castrated 3-15 months were divided into several groups. Two control groups ovariectomized 15 months and 3 months to one year respectively were not injected with hormones. To a group ovariectomized 3 months and to another ovariectomized 15 months, 240 International Units of estrone were administered by subcutaneous injection in 10 doses over a period of 5 days. To a fourth group ovariectomized 3 months, testosterone propionate was administered subcutaneously in a total quantity of 50 mgm. divided into 10 doses. For the purpose of comparison, a further group of normal animals in estrus and diestrus was included. At the end of the treatment period the rats were killed by a blow on the head and the uteri removed. The tubal half of one horn of each uterus was suspended in 30 cc. of Locke's solution at 37.5°C. Contractions were recorded in the usual manner. The amount of pitocin employed was 0.1 unit in all experiments.



## RESULTS

The summarized results are presented in table 1. The changes in rate, amplitude, tone and duration of contractions were obtained from measurements made from the kymograph records. The figures given in table 1 are medians together with percentile changes in activity brought about by the addition of pitocin.

TABLE 1

EXPERIMENTAL GROUPS	NUMBER OF ANIMALS	RHYTHM		RATE OF CONTRACTIONS (PER MIN.)			AMPLITUDE OF CONTRACTIONS (MM.)			TONE (MM.)			DURATION OF CONTRACTIONS (SEC.)						
		Before pitocin	After pitocin	Before pitocin	After pitocin	Percentile change	Before pitocin	After pitocin	Percentile change	Before pitocin	After pitocin	Percentile change	Before pitocin	After pitocin	Percentile change				
Normal rats in diestrus	3	Regular	Regular	1.6	1.7	6.2	35	30	-14	20	30	50	30	30	0				
Ovariectomized 3 months to 1 year, no treatment	5	Regular	Regular	1.38	1.5	8.7	40	60	50	38	62	63	50	45	-10				
Ovariectomized 15 months, no treatment	3	Regular	Regular	2	2	0	3	3	6	9	37	36	-2	7	6.7				
Normal rats in estrus	5	Irregular	Regular	1	1.5	50	17	45	165	25	35	40	30	55	83				
Ovariectomized 15 months, 240 I.U. estrogenic substance in 10 doses	2	Regular	Regular	0.93	1.0	5.3	7	6	11	45	23	5	24	2.1	-37				
Ovariectomized 3 months, 240 I.U. estrogenic substance in 10 doses	10	Irregular	Regular	0.8	1.4	75	97	5	99	1.56	13	24	85	49	51	4.1			
Ovariectomized 3 months, 1 mgm. diethylstilbestrol in 10 doses	7	Irregular	Regular	0	66	1.3	97	60	62	3.3	18	30	67	34	43	26			
Ovariectomized 3 months, 50 mgm testosterone propionate in 10 doses	7	Irregular	Irregular	0.85	0	0.03	9	4	6	6	14	3	117	12	12	0	15	25	67

*Animals castrated 15 months*

These uteri were quite pale, small and almost thread like. The uterine tissue exhibited weak spontaneous activity and the response to pitocin was slight. The uteri of two similar castrates given estrone showed a greater amplitude of contractions than those of the castrated untreated animals. These tissues responded to pitocin by a considerable increase in the amplitude of contractions and a rather marked decrease in their duration.

*Animals castrated 3 months to one year*

These uteri were pale and small, but were not as atrophic as those of animals castrated 15 months. Uteri of animals castrated three months to one year and given no hormone treatment showed a greater degree of spontaneous



activity than similar tissue from rats castrated 15 months and the response to pitocin was proportionately greater. The amplitude of contractions was quite markedly increased after pitocin and the duration of contractions was shortened. The behavior of the myometrium simulated that of the normal animal in diestrus (fig. 1, a, b).

Fig. 1a

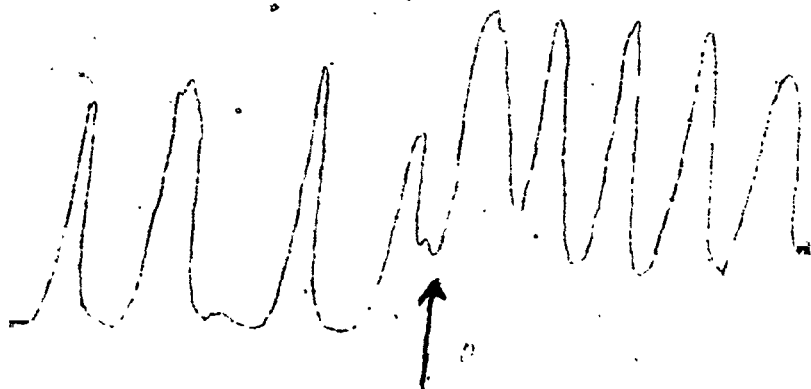


FIG. 1. Kymograph records of contractions of the uteri from the following animals: (a) Normal animal in dioestrus. (b) Rat castrated 3 months without hormone therapy. (c) Normal rat in estrus. (d) Rat castrated 3 months, given 240 I.U. estrogenic substance. (e) Rat castrated 3 months given 1 mgm. diethylstilbestrol. (f) Same as "e." (g) Rat castrated 3 months given 50 mgm. testosterone propionate.

The arrow marks the point at which 0.1 unit of pitocin was applied.

*Animals castrated three months and pre-treated with estrogenic substance*

These uteri were hyperemic and appeared much like those of the normal animal in estrus. Uteri of these animals showed an irregular rhythm before pitocin was applied; after pitocin the rhythm became regular. The rate of contraction was quite slow (median 0.80 per minute) and became quite markedly increased after pitocin was applied (1.4 median); there was also quite a marked increase of tone. The response of the uteri of these animals compared to that of normal animals in estrus, showed a smaller increase in amplitude of contractions subsequent to the use of pitocin (fig. 1, c, d).



Fig. 1b

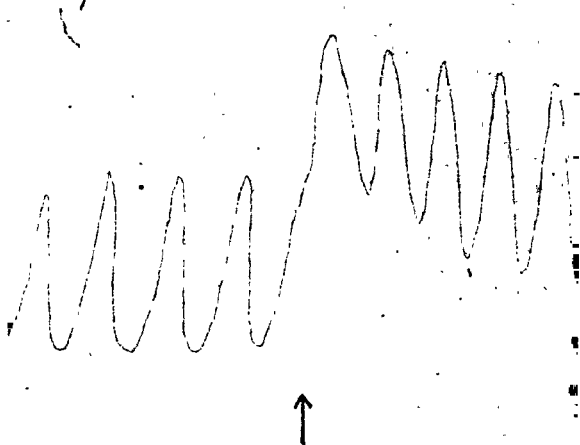


Fig. 1c





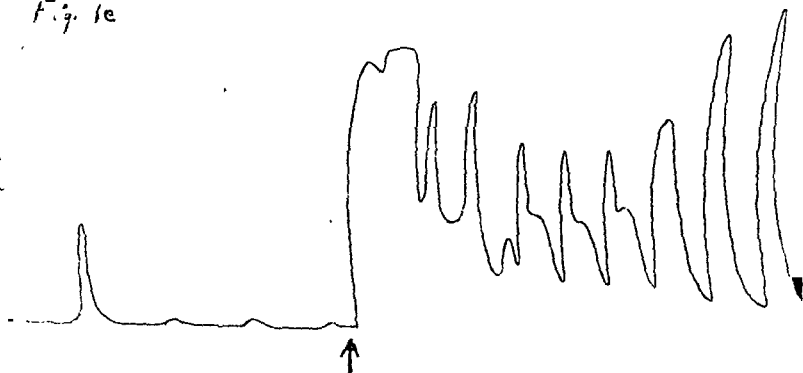
*Animals castrated three months and given one mgm. of diethylstilbestrol*

The uteri were markedly enlarged and hyperemic. They showed an irregular spontaneous rhythm which became regular after pitocin was introduced. The rate of contractions was slow initially (median of 0.66 per minute) but showed quite a marked increase after pitocin (median 1.3 per minute).

*Fig. 1d*



*Fig. 1e*



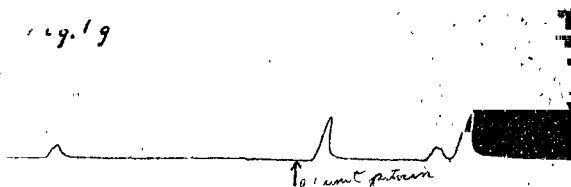
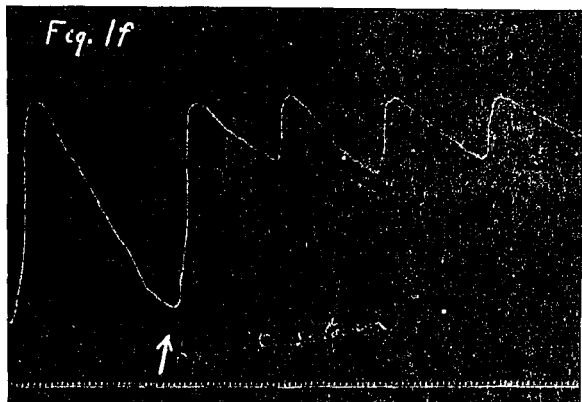
(See Figure 1, a for legend.)

(fig. 1, e). It may be seen from these results that the synthetic estrogen, diethylstilbestrol, restores the contractility of the uterus of the ovariectomized animal and enables it to respond to pitocin in much the same way as does the uterus of the normal estrus animal. However, the former exhibited a tendency to become spastic after pitocin was applied (fig. 1, f).



*Animals castrated 3 months and given 50 mgm. of testosterone propionate*

The uteri of these animals were large, pale and filled with fluid. The following phenomena were observed in these tissues: The rhythm was normally irregular and the contraction rate was slow. After pitocin was applied there



(See Figure 1, a for legend.)

was no increase in tone, the rhythm became even more irregular and there was a marked increase in amplitude and duration of contractions (fig. 1, g).

#### DISCUSSION

The results obtained from the estrone treatment of animals castrated 15 months showed that the uterine tissue retained to a considerable extent the



capacity to respond to that hormone even after a rather prolonged period. However, there was not a complete restoration of the normal pattern of uterine motility and we do not know whether this would be reestablished by longer treatment with the estrogen.

The myometrium of animals castrated for a shorter time (3 months to one year) showed a greater degree of spontaneous motility than similar tissue from animals castrated 15 months. The records of the estrogen-treated animals show the various types of contractions distinguished by Harne (2) in his study of the excised uterus of the rat at various stages of the estrus cycle. Furthermore, we were able to confirm his findings that the oxytocic posterior pituitary hormone converts submaximal to maximal contractions. We observed what he describes as circular contractions in a number of the records, especially those obtained from the uteri of testosterone treated animals.

The results of the experiments on diethylstilbestrol demonstrate that the uterus of the castrate animal responds to it in much the same way as to estrone. There were, however, some qualitative differences. Especially noteworthy is the tendency of the uterus of the diethylstilbestrol-treated animal to become spastically contracted after pitocin was applied. These experiments show that diethylstilbestrol given to the castrate animal will restore uterine motility to almost the normal pattern. Our results in these experiments may be contrasted with those of Dawson and Robson (3) who studied the effects of the application of diethylstilbestrol to various tissues. They found that this synthetic estrogen had a depressant effect when applied to uterine and other smooth muscle *in vitro*.

The gross appearance of the uteri of castrates injected with testosterone deserves some comment. These uteri were large, pale and contained much fluid. Apparently the response of the castrate rat uterus differs from that of the same tissue of animals with intact ovaries following the administration of testosterone. Brooksby (4) administered 500 gamma of testosterone daily for 10 days to rats immediately after ovariectomy and found that the uteri were enlarged and hyperemic. We were struck by the complete lack of hyperemia of the uteri of the testosterone-treated animals in our series. McKeown and Zuckerman (5) found that the histological reaction of the uteri of ovariectomized animals to testosterone is quite different from that obtained in the animal with intact ovaries.

The pattern of motility and the response to pitocin of the myometrium of castrate rats was quite markedly modified by testosterone treatment. The irregular rhythm, absence of tone change and the continuance of the irregular rhythm after pitocin shows that this androgen markedly alters the functional activity of the uterine tissue of castrate animals. Several investigators have also found that testosterone has an inhibitory action on uterine muscle. Robson (6) using segments of surviving rabbit uterus and Rubin and Davids



(7) employing the carbon dioxide insufflation method in the intact rabbit observed a marked inhibitory effect on the tubal muscle with a diminution of general tonicity and amplitude of contractions. Wilson and Kurzrok (8) administered testosterone to a human patient and observed that it produced a type of uterine motility which resembled that seen during the luteal phase of the estrus cycle.

#### CONCLUSIONS

1. The uteri of animals spayed 15 months showed slight spontaneous motility and do not respond to pitocin in a normal manner.

2. Pre-treatment of similar animals with estrone caused only a slight degree of recovery of spontaneous activity and of the ability to respond to pitocin.

3. The uteri of animals castrated 3 months to 1 year show a pattern of motility resembling that of uteri of normal animals in diestrus.

4. The uteri of animals castrated 3 months can be made to respond quite normally to pitocin by adequate pre-treatment with estrone.

5. Diethylstilbestrol treatment of the castrate animal will restore uterine motility to almost the normal pattern; the uteri of these animals tended to become spastic after pitocin was applied.

6. The myometrium of the castrate animal given testosterone showed an altered functional activity and the response to pitocin consisted in a marked increase in the amplitude and duration of contractions.

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# ACTIONS OF CURARIZING PREPARATIONS IN THE HUMAN

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Received for publication July 3, 1941

A recent revival of efforts to obtain a satisfactory curarizing agent has been stimulated by two possible uses of such a substance in clinical medicine. The first application has been in the treatment of patients suffering with spasticity and with extra-pyramidal types of rigidity. Bremer (1), and Bremer and Titeca (2) have shown that some forms of crude curare reduce tonic muscular contraction while at the same time permitting active phasic contractions to continue. Bremer suggested that the selective action of curare on tonic contraction is due to the blocking of repetitive responses. Rapid trains of tonic impulses are rendered ineffectual in exciting muscle responses, while the response to short bursts of impulses in phasic contractions is relatively unaltered. West (3), however, tested a large number of curare-like alkaloids and concluded that the selective action of crude curare on various forms of spasticity is due to a central effect. He presented evidence that the relief of spasticity in certain patients is not associated with the true curare action at the neuromuscular junction. West obtained prolonged reduction of rigidity in a number of spastic patients whom he treated with certain curare preparations.

Burman (4) also used crude curare as well as erythroidine in the treatment of spasticity and athetosis, and reported reduction of muscle tone for periods of days following a single injection. The success of these investigators has suggested a large field of usefulness for drugs with the ability to reduce spasticity, and encourages the search for a safe, long-acting, easily obtainable substance having a curariform action.

Recently, curarizing agents have been found useful in reducing the severity of the convulsions observed in Metrazol shock therapy. Bennett (5) has found that by preliminary administration of curare, or quinine methochloride, it is possible to produce sufficient muscular paralysis to reduce materially the severity of metrazol convulsions. Similar results have been obtained by the use of Beta erythroidine. It thus appears that a short-acting, safe, curarizing agent might prove to be a valuable adjunct to this form of shock therapy.

In view of these therapeutic possibilities, it seemed advisable to study in more detail the action of the currently available curarizing agents, in an effort



to compare their safety and relative advantages and disadvantages for use in the human patient.

Three substances known to be curarizing agents have been used: The first of these, quinine methochloride, is a substance prepared from quinine by substitution of a methyl group on the tertiary nitrogen atom of the quinuclidine ring. A quarternary ammonium base is thus formed which has been shown by Harvey (6) to have the typical curare action on neuromuscular transmission. Beta erythroidine (Merck) is an alkaloid now available in purified form. It has been shown by Unna (8) to have a curare-like action. "Unauthenticated" curare (Squibb) is an extract of crude curare standardized and prepared for clinical use.

The common property possessed by all three of these drugs is known to be their effect on neuromuscular transmission. The characteristic changes in muscle response under conditions of curarization in animals have been studied with curarine by Brown (7), with erythroidine by Unna (8), and with quinine methochloride by Harvey (6). They consist primarily of an inability of the muscle to maintain a sustained tetanus when the nerve is stimulated at high frequencies, although the response evoked by direct stimulation of the muscle is not altered. Following larger doses of curarizing agents there is eventually an inability of the muscle to respond to even a single nerve impulse.

When the electromyogram is studied the failure of the muscle response during tetanization of the motor nerve is again the striking abnormality. Whereas normally the motor nerve may be stimulated repeatedly at frequencies up to 60 per second with little alteration in the resulting muscle action potentials, the action potential of the curarized muscle undergoes a progressive diminution in size during repetitive stimulation of the nerve. Under these conditions one finds that although the electrical response of the muscle to a single nerve shock may be normal, there follows a period of depression during which the response to a second nerve impulse is much reduced.

A satisfactory method for recording quantitatively the muscle action potentials in the human has recently been reported (9) and provides a means of observing changes following curarization which are similar to those seen in animal experiments. In the human, the action potential of the *abductor digiti quinti* muscle may be recorded through the skin by means of electrodes placed over the belly and the tendon of this muscle. On supramaximal stimulation of the ulnar nerve under these conditions an action potential is recorded which is quite comparable to that of an exposed muscle, and which undergoes changes similar to those seen in animal experiments during curarization.

The present investigation has been centered about the use of this method to study curarization in the human. By observing changes in the muscle action potential in response to nerve stimulation, it has been possible to estimate the actual degree of curarization and to correlate this with the clinical signs ordinarily noted.



Studies have been made in this fashion with each of the three available curarizing agents. The administration of these substances clinically has been by intravenous injection. In most instances the drug has been given slowly while observations were made on the patient's appearance, pulse rate and blood pressure, and while the state of neuromuscular conduction was checked continually by observation of the muscle action potentials following stimulation of the ulnar nerve. The injection was continued slowly until the desired stage of curarization was reached. As long as the administration was slow, the procedure was safe, for with termination of the injection, there was rapid disappearance of drug effect.

A total of 35 injections have been administered to 23 patients including the following cases: Spastic diplegia 5, dystonia 1, parkinsonism 3, amyotrophic lateral sclerosis 2, cord tumor 1, involutional melancholia 3 (metrazol), tabes dorsalis 1, brain tumor 1, Schilder's disease 1.

### RESULTS

The clinical picture of curarization is now well known, and is identical for the three drugs tested. Weakness first involves the eyelids and extra ocular muscles. The lids become heavy and cannot be kept open. Diplopia develops, and it becomes impossible for the patient to maintain lateral or upward gaze. The ptosis increases until the eyelids are closed, the face becomes expressionless, speech is thick and nasal, and swallowing difficult. At this stage it is difficult, and soon impossible for the subject to raise the head from the pillow. Most patients state that they feel as though the head were heavy, or as though something were holding it down to the bed.

Little effect on somatic musculature is evident, until marked bulbar signs are present. However, by the time the patient is unable to raise the head, he usually also complains of general bodily weakness and a feeling of fatigue. At this stage muscle strength is found to be reduced. If administration of the drug is continued, it becomes impossible for the patient to raise the legs from the bed, and general body relaxation develops. We have not carried any patient to the point of complete inability to move, nor have we observed respiratory difficulty, although both these conditions may be effected in animals if large dosages are employed.

Exact comparison of the dosages of the three drugs cannot be established, as the total amount required varies greatly with the patient and with the rapidity of injection of the drug. Generally speaking, the stage of marked bulbar signs, with moderate involvement of general bodily musculature, may be reached with quinine methochloride, 8 to 12 mg./kg. given over a period of 5 minutes, with erythroidine 15 to 17 mg./kg. given over a period of 5 minutes, and with curare 1 to 2 mg./kg. given over a period of 2 minutes.

Changes in the electromyogram follow the clinical course of the curarization. There is usually little visible alteration in the electrical response of the



*abductor digiti quinti* muscle to stimulation of the ulnar nerve until a moderate degree of weakness of the muscles innervated by cranial nerves has been reached. At this point it is first observed that, when the nerve is tetanized at frequencies of about 50 per second, there develops a progressive decline in the amplitude of the resulting muscle action potentials. As symptoms progress and the patient becomes unable to raise his head, this abnormality becomes evident with much slower rates of stimulation (10 per second), and, at this stage, is sufficiently pronounced to be appreciable as a diminution in the amplitude of the second of a pair of responses separated by intervals up to one second. If administration of the drug is continued and the patient notices generalized muscular weakness, there now occurs a progressive decline in the amplitude of the response to a single nerve stimulus. We have carried curarization to the point at which the greatest action potential obtainable, in response to a single supra maximal nerve stimulus, was but 40 per cent of the potential prior to administration of the drug.

Following cessation of the injection there is rapid decline in the drug effect. With quinine methochloride return of power commences as soon as the injection is stopped, and by the end of 10 or 15 minutes, full strength has usually returned. Similarly with erythroidine, the effect is fleeting, and passes off rapidly. With both these drugs, the removal, by whatever means it takes place, is so prompt that unless the rate of injection is fairly rapid, removal may keep pace with injection and prevent a full effect.

The action of curare is a little more slow in developing, and the full effect is usually not noted until about five minutes after the injection is complete. The duration of its effect is also longer, and symptoms may persist for as long as twenty minutes. None of the drugs produces significant curarizing effect for more than an hour after the end of the injection.

The actual curarizing effect described above, is the only reaction, common to all three drugs, which is always observed following their administration. Other effects noted are as follows:

*Cerebral action.* We have not noted any marked central effect from curare extract. Erythroidine and quinine methochloride frequently produce unquestionable mental changes. With quinine methochloride the patients often become drowsy and listless. Complaining of ptosis due to the curarizing action of the drug, they also affirm that they are sleepy. Frequent yawning is often a prominent symptom. The mental reactions become slow, and in one or two instances, apprehensive patients have appeared slightly confused. Similar reactions occur with erythroidine. One patient, suffering from a spastic diplegia (wt. 50 kg.), was given erythroidine and had a severe mental reaction. As the administration of the drug was started, he yawned frequently, and complained of being drowsy. He seemed confused, and would not respond to commands. A total of 750 mg. of erythroidine was given over a period of 6 minutes. For the ensuing half hour, the patient was irrational



and disorientated as to time and place. He did not respond to questioning, and his speech was incoherent. This picture gradually cleared up, but the patient continued to be unsteady on his feet and to complain of dizziness until about one hour after the injection. The outstanding characteristics of this episode were confusion and disorientation.

Although some evidence of this type of reaction is frequently observed, many patients show no cerebral effect whatever, and remain entirely normal throughout the entire injection.

We have attempted to demonstrate the cerebral action by studying changes in the electroencephalogram during the administration of curare, erythroidine, and quinine methochloride. In several instances during the recording, mild mental confusion occurred, but in no case was it accompanied by any distinct change in the electroencephalogram. The alpha rhythm remained unchanged in occurrence, amplitude, and rate, and no delta activity was observed.

*Circulatory effects.* Following the administration of any one of the three drugs tested, there was a moderate fall in blood pressure and slight elevation in pulse rate. With curare, this usually amounted to a fall in systolic pressure of about 5 to 10 mm.Hg and a 5 per cent rise in pulse rate. With erythroidine, the fall in blood pressure was a little greater, being from 10 to 15 mm.Hg, with a 15 per cent rise in pulse rate. The greatest fall in blood pressure occurred with quinine methochloride, and amounted to 20 mm.Hg with a corresponding increase in pulse rate. In no case was the fall in blood pressure rapid, or sufficiently marked to become alarming. In every instance the pressure returned to normal levels when administration of the drug was discontinued.

Accompanying the fall in blood pressure there was often a distinct change in the appearance of the patient. This was evident with erythroidine and quinine methochloride but was not seen in patients receiving curare. It was characterized by pallor, sweating, and questionable cyanosis. The appearance resembled that of individuals in shock. It is probable that this change is due to circulatory disturbances. The development of this appearance was very common following the two drugs mentioned, and was most noticeable following large doses given rapidly. This reaction was also a fleeting one, and passed off as the curare effect diminished.

In view of these obvious (though minor) circulatory effects, an attempt was made to demonstrate changes in cardiac output during curarization. Through the courtesy of Dr. Isaac Starr, ballistocardiographic records were obtained and cardiac outputs were determined, during curarization with each of the three drugs, in dosage sufficient to prevent the patient from being able to raise the head from the pillow.

Changes in cardiac output under these conditions were extremely slight. Those following curare (45 mg. in 3 minutes in a patient weighing 42 kg.) and quinine methochloride (135 mg. in 5 minutes in a patient weighing 30 kg.) were so small as to be within the limits of error of the method. Following erythroi-



dine, (800 mg. in 3 minutes, weight of subject 42 kg.), the cardiac output fell from 13 cc./kg./minute to 10 cc./kg./minute.

*Toxic reactions.* The most alarming toxic reaction was that occasionally observed after quinine methochloride. In 13 injections of this drug a severe gastro-intestinal reaction has occurred twice. In each case the patient received the injection, had the typical curarizing reaction, and recovered from it rapidly. About a half hour after the end of the injection, at a time when the curare effect had completely disappeared, the patient began to complain of backache, abdominal cramps, faintness, and nausea. This was followed by diarrhea and vomiting. Severe abdominal pain lasted for about a half hour, then gradually subsided. Both patients were quite well at the end of two hours. During this period, there was no change in blood pressure. There was no fever or leucocytosis. The urine was entirely normal at this time, and never showed any abnormality. Although we have no evidence as to the fundamental nature of this reaction, the time of its occurrence indicates that it is not associated with any curare effect but that it is probably due to a reaction to the quinine molecule.

Respiratory embarrassment has not occurred in patients receiving quinine methochloride or erythroidine. West (10) has reported bronchial spasm following the use of curare extracts, and we have occasionally observed mild respiratory difficulty with the curare extract we have used. The patients appeared to have difficulty in inhaling rather than in exhaling. The reaction, though alarming, has proven transitory, and has cleared up rapidly after the administration of adrenalin. The effect of the latter medication may merely have been incidental, but the response suggested that it may have had a beneficial effect in relieving respiratory embarrassment.

*Relief of spasticity.* The relief of spasticity observed in patients suffering from various diseases has not been striking. With full curarization there was always marked diminution in muscle tone, but in most instances relaxation has occurred only when bulbar signs were so marked that the patient could not lift his head and could barely talk, chew or swallow.

Furthermore, the action of all three of the curarizing agents, when they are given intravenously, is of very short duration. With none of the drugs has the typical bulbar paralysis persisted for more than three-quarters of an hour after the end of the injection.

In our experience, no patient has felt more comfortable during the injection than he was under normal conditions. This applies to patients with pyramidal and extrapyramidal rigidities as well as athetosis and dystonia. However, it has been volunteered by several of the patients that for 1 to 2 days after the injection, there appeared to be a little less stiffness than was previously the case. This has been mentioned by patients who have received any of the three drugs. Although objectively we could observe little change in the patients, the frequency of the report has made us a little hesitant to disregard it entirely.



*Prevention of injury during metrazol convulsions.* For this purpose the curarizing substance to be employed was given as rapidly as possible in full dosage, and was followed at the height of the curarizing effect by the injection of metrazol. Technical difficulties have prevented action potential recording during this procedure, but the degree of curarization attained has been followed by clinical observation.

The results indicate that all three substances are capable of reducing the severity of the metrazol convulsion. Large dosage is required, and we have been surprised to find that individuals so curarized that they are unable to raise the head may still have a strong metrazol convulsion with board-like abdomen and rigid back. Strong convulsions have been observed in a patient weighing 70 kg. following 1500 mg. of erythroidine given in  $2\frac{1}{2}$  minutes, and in the same patient after 520 mg. of quinine methochloride given in  $3\frac{1}{2}$  minutes. Following both of these injections the patient appeared well curarized and could not raise his head or his legs from the bed. The convulsions, however, were severe.

With curare, the relaxation appeared to be more complete, and although the degree of curarization clinically appeared the same, with curare the convulsions were usually much less severe than with either of the other drugs.

#### DISCUSSION

In our hands, curarizing substances have not proven beneficial in the treatment of spastic patients. When the drugs are given intravenously, the resulting bulbar palsy is so disturbing that the slight relief of spasticity obtained is not appreciated by the patient. Furthermore, with relaxation of the spastic muscles, the patient becomes weak and for this reason voluntary movement is increased little if at all.

The possibility still exists that a longer acting curare-like substance, particularly one which is stable enough to be effective by mouth, might be helpful in treating spastic states. An effect might be obtained with long continued administration to the point of mild curarization which is not evident in these acute experiments when the drug is given intravenously.

We are unable to account for the lasting relief which several patients have reported. In a suggestible group of patients such as this, subjective impressions must be viewed with caution. The possibility presents itself that the persistent action may be due to some central effect. This opinion has also been expressed by West (10).

The action potential studies which are recorded show that the fundamental effect of these three drugs on the neuromuscular junction is entirely similar. The long lasting depression which follows a single nerve stimulus has the same time course regardless of which of the drugs has been administered.

All three drugs are capable of reducing the severity of metrazol convulsions. In general, we believe that the curare extract which we have used is probably



most satisfactory. The general appearance of the patient is better, fall in blood pressure is slight, and curarization, for some reason, more effective. It has the disadvantage of producing occasional respiratory embarrassment.

We wish to thank the Squibb Company for generous supplies of curare extract and quinine methochloride and the Merck Company for the erythroidine used in these experiments.

#### SUMMARY

The actions of curare extract, erythroidine, and quinine methochloride have been compared in the human subject.

All three drugs produce a characteristic clinical picture accompanied by typical changes in the electromyogram.

Mental disturbances characterized by confusion and disorientation occasionally occur with erythroidine and quinine methochloride.

Minor circulatory changes have been observed and appear more severe with quinine methochloride and erythroidine than with curare.

Other toxic manifestations have been described.

Relief of spasticity has been slight, of brief duration, and has not appeared until there was considerable weakness of the extraocular, facial, and pharyngeal muscles.

The severity of metrazol convulsions has been decreased.

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## STUDIES ON A SERIES OF NEW TRIAZOLE DERIVATIVES<sup>1, 2</sup>

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Received for publication July 30, 1941

Many reports concerning the physiological properties of compounds containing a triazo group, such as cyclohexyethyltriazol ("azoman" or "triazol 156"), are concerned with their stimulant or convulsant action (1, 2, 3, 4, 5). This suggested the possibility that certain new triazole compounds (table 1) which had been made available<sup>3</sup> to us might possess similar properties, but subcutaneous injections of 20 to 120 mgm. per kilogram of body weight<sup>4</sup> of the sodium salt and the dimethyl compound produced little or no apparent effect in rats. Conclusive data following parenteral administration of the other members of the present series could not be obtained because of their low order of solubility. Larger doses of the dimethyl and the diethyl triazoles given in acacia suspension to rats by stomach tube failed to produce convulsant effects but did reduce normal rectal temperature 6° to 8°F.

*Antipyretic effect.* The foregoing temperature effects suggested a study of the present series of compounds in animals with fever. The average normal rectal temperature in groups of four or more rats (as indicated in figure 1) weighing between 170 and 225 grams was determined from three or four readings on each animal taken over a period of 8 hours. This composite figure was between 98.6° and 100.1°F. These rats then were made febrile by the method of Smith and Hambourger (6) which depends upon subcutaneous injection of 10 cc. per kilogram of a 15 per cent suspension of dried finely powdered yeast. After about ten hours when the febrile reaction was maximal (average 2.5°F.), the compounds to be tested were suspended in 2 per cent acacia solution in such concentration as to contain the desired dose in 10 cc. per kilogram and then were administered orally by means of a small rigid stomach tube attached to a syringe.

<sup>1</sup> Presented before The American Society for Pharmacology and Experimental Therapeutics, Inc., Chicago Meeting, April, 1941 (THIS JOURNAL, 72: 10, 1941).

<sup>2</sup> This investigation was supported in part by the Smith, Kline and French Fellowship and The D. J. McCarthy Foundation.

<sup>3</sup> These compounds were made available by Professor R. L. Shriner, of the University of Illinois.

<sup>4</sup> Throughout the remainder of the text it is understood that all doses mentioned are per kilogram of body weight.



Figure 1 shows the effects of single doses of 100 mgm. of the triazole derivatives (except dipropyl) and of aminopyrine. It is to be observed in this figure that fever in the 20 yeast-treated control rats, which received only 2 per cent acacia solution, was well maintained throughout a 24 hour observation period. The similar fever in the animals which received aminopyrine, however, was promptly reduced to normal, while in those animals which received dimethyl and diethyl triazole a reduction of more than 2° below the normal level was obtained. This subnormal temperature rose gradually to normal within 5 hours. The sodium salt exhibited antipyretic activity but was definitely less

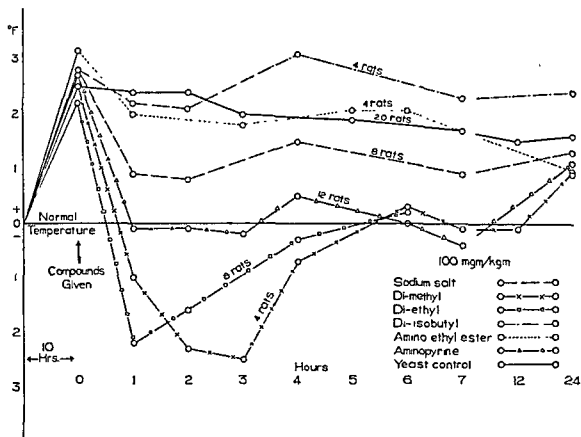
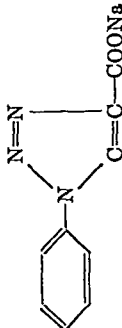
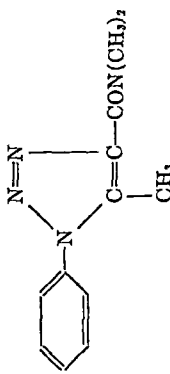
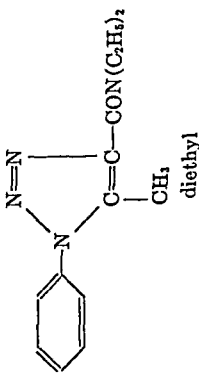


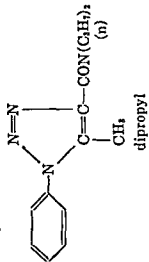
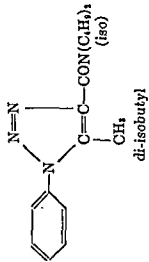
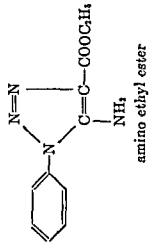
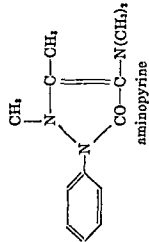
FIG. 1. COMPARATIVE ANTIPYRETIC ACTIVITY OF TRIAZOLE DERIVATIVES AND AMINOPYRINE

effective than the dimethyl or diethyl derivatives. The di-isobutyl and amino ethyl ester triazoles were without appreciable antipyretic action in the above dose. Insofar as other dose ranges are concerned it may be stated that the di-isobutyl compound was inactive in doses as large as 200 mgm., whereas the diethyl triazole and aminopyrine produced slight but definite antipyretic effects of about equal intensity in doses as low as 25 mgm. The other compounds were ineffective in this low dose. None of the rats used in the antipyretic studies exhibited either acute or delayed symptoms of toxicity. At the site of the yeast injection there usually developed an abscess which healed after ten or twelve days.



COMPOUNDS		ANALGESIA IN CATS					TOXICITY IN RATS	
Name	Formula and abbreviation	Dose (oral)	Number showing analgesia/number used	Average increase in threshold pain stimulus	Con-vul-sions	Deaths	Dose (oral)	Number deaths/number used
		mgm. per kgm.		per cent			mgm. per kgm.	
1 Phenyl-5 methyl-1,2,3 triazole-4 sodium carboxylate	 sodium salt	100	4/4	411	4	2	2000	4/4
		75-80	3/3	100	3	1	1500	5/8
		50-60	4/7	154*	1		1000	0/4
1 Phenyl-5 methyl-1,2,3 triazole-4 dimethyl carboxamide	 dimethyl	500	1/1	Coma	1	1	2000	4/4
		100	2/2	Coma	2	2	1000	4/4
		75	3/4	Coma	2	2	750	8/8
		50	4/6	86	2	1	500	4/8
		35	0/1	0	0		250	0/4
		25	0/1	0	0			
1 Phenyl-5 methyl-1,2,3 triazole-4 diethyl carboxamide	 diethyl	150	1/1	130	1	2	1000	4/4
		100	3/3	210	3		750	3/4
		75	3/4	175	3		500	5/8
		50	4/5	140	3		250	0/4
		35	4/4	100	0			
		25	0/2	0	0			



1 Phenyl-5 methyl-1,2,3 triazole-4(n) dipropyl carboxamide		500 250 100 50	1/1 1/2 0/1 0/1	Coma 36 0 0	1 1 0 0	1	1500 500	0/1 0/1
1 Phenyl-5 methyl-1,2,3 triazole-4 di-isobutyl carboxamide		1000 500 100	0/3 0/3 0/1	0 0 0	0 0 0		2000 1000	0/4 0/4
1 Phenyl-5 amino-1,2,3 triazole-4 ethyl carboxylate		300 200 100	0/1 2/2 2/3	0 70* 150*	0 1 1		2000 1000	0/4 0/4
Dimethylamino phenyl dimethylpyrazolon		225-250 200 100 75-80 50 35	2/2 2/2 2/3 6/7 2/4 0/2	200 60* 160 73 50 0	2 0 1 0 0 0	2 1	2000 1500 1000 750 500	5/6 8/8 14/29 1/8 0/2

\* Analgesia variable



*Analgesic effects.* Since many common antipyretics also possess analgesic action the effect of the triazole compounds upon the threshold pain stimulus in cats was studied by a method similar to that described by Eddy (7). The present method consisted of applying a slowly increasing pressure to the distal two inches of a cat's tail by means of a graduated lever along which a weight was moved. The minimal amount of pressure necessary to elicit signs of pain was taken as the threshold pain stimulus. An increase of more than 25 per cent in threshold pain stimulus after administration of a given compound was regarded as evidence of analgesia because variations from the mean during control periods did not exceed 25 per cent.

The analgesic effects of the present triazole derivatives (sodium salt, dimethyl, diethyl, dipropyl, di-isobutyl and the amino ethyl ester) were compared with those of aminopyrine in 86 cats. All compounds were given orally in capsules and the data are summarized in table 1, in which it may be noted that the amino ethyl ester derivative caused only slight or extremely variable analgesic effects in doses as large as 300 mgm., while the di-isobutyl compound was devoid of any such effect even with doses of 1 gram. The lowest effective dose of the dipropyl compound was 250 mgm. but a 500 mgm. dose produced a condition of coma which made the measurement of pain threshold impractical. The dimethyl compound was not effective in doses below 50 mgm. and the effects of higher doses (75, 100, 500 mgm.) could not be measured because of coma. The sodium salt was used in doses ranging from 50 to 100 mgm. with a marked variation in results up to a 400 per cent increase in pain threshold. The diethyl compound in the minimal effective dose of 35 mgm. caused, with no apparent indication of toxic effects, an increase of 100 per cent in pain threshold. Marked analgesia was obtained also with higher doses of this compound but increasing the dose to 50 mgm. caused convulsions in 3 out of 5 animals, whereas 2 out of 3 died after a dose of 100 mgm. It is to be noted that aminopyrine required between 75 and 100 mgm. to produce a depth of analgesia comparable with that of the minimal effective dose of the diethyl compound, and that the upper limit of this dose range caused convulsions and death in 1 of 3 animals (table 1). Other untoward symptoms such as restlessness, salivation and muscular incoordination were sometimes seen after the triazole compounds. Aminopyrine in all doses used caused profuse salivation and rapid panting respiration.

*Toxicity.* The compounds were suspended in 2 per cent acacia solution and administered by a rigid stomach tube; four or more rats were used for each dose. Determined in this way the L.D. 50 (table 1) was 1500 mgm. for the sodium salt, 500 mgm. each for the dimethyl and diethyl compounds and more than 2000 mgm. for both the di-isobutyl and the amino ethyl ester derivatives. The amount of the dipropyl compound available was not sufficient for us to obtain definite data on its toxicity. The L.D. 50 of aminopyrine in these tests was 1000 mgm. which is in fair agreement with the report of Brownlee



(8) who found a comparable figure of 1150 mgm. The few casual observations concerning toxicity in cats would indicate that they are more susceptible to these compounds than rats. This may be due to the difference in method of administration or some other cause which has not been studied.

**DISCUSSION.** Comparison of the temperature curves (figure 1) indicates that in 100 mgm. doses the dimethyl and diethyl triazoles exerted a more profound effect than aminopyrine upon the temperature regulating mechanism, but too great a significance should not be attached to this observation, because in smaller doses these compounds were found to possess no greater antipyretic action than aminopyrine.

The sodium salt and the dimethyl and diethyl amides had more powerful analgesic effects than the other members of the series, but definite analgesia without marked toxic symptoms was obtained only with the diethyl derivative. The margin of safety of this compound was narrow because convulsions were produced by doses slightly above the minimal analgesic level and a three-fold increase of this dose caused death in two thirds of the animals. Discrepancies were noted in the degree of analgesia produced by different doses. For example, 50 to 100 mgm. sometimes decreased the response to pain to a greater extent than larger doses. A possible explanation of this may lie in the untoward side effects such as convulsions or other nervous disturbances which may militate against analgesia unless the dose is large enough to cause collapse. Similar variations were seen also after aminopyrine.

Because of greater toxicity, narrow margin of safety, untoward side reactions or low order of activity none of this series of triazole compounds, on the basis of our present information, possesses any distinct advantage over aminopyrine as an antipyretic or analgesic drug.

#### SUMMARY

1. The antipyretic and analgesic effects of six derivatives of 1-phenyl, 1,2,3-triazole 4-carboxylic acid were compared with that of aminopyrine.

2. 1-phenyl 5-methyl, 1,2,3-triazole, 4-diethyl carboxamide and the corresponding dimethyl derivative were active antipyretics in rats. The sodium salt of 1-phenyl, 5-methyl, 1,2,3-triazole, 4-carboxylic acid and the ethyl ester of 1-phenyl, 5-amino, 1,2,3-triazole, 4-carboxylic acid were less efficient antipyretics than the dimethyl and diethyl amides while the di-isobutyl amide was devoid of antipyretic action.

3. The diethyl compound in a nontoxic dose doubled the pain threshold stimulus in cats and was more effective than aminopyrine. The other triazole derivatives in nontoxic doses were less effective than aminopyrine.

4. The dimethyl and diethyl triazoles were twice as toxic in rats as aminopyrine whereas the other members of the present series were less toxic than aminopyrine.

5. Thus far no advantage over aminopyrine has been found for these compounds.



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# THE PHARMACOLOGY AND TOXICOLOGY OF THE ETHYL ESTER OF 1-METHYL-4-PHENYL-PIPERIDINE-4-CARBOXYLIC ACID (DEMEROL)<sup>1</sup>

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Received for publication July 31, 1941

In 1939 Eisleb and Schaumann (1) reported the synthesis of the ethyl ester of 1-methyl-4-phenyl-piperidine-4-carboxylic acid. Because of the high alkalinity and low water solubility of this compound it is prepared for experimental and clinical uses in the form of the hydrochloride. This has been given the names Dolantin, Dolantol, D-140 and Demerol. The water solution of this drug is slightly acid and has a bitter taste. For the sake of brevity this compound will be referred to as "Demerol" throughout this paper. Eisleb and Schaumann report that this compound in a 1:5,000,000 dilution will antagonize the effects of acetylcholine on the isolated gut, that it will partially inhibit the effects of acetylcholine on the blood pressure, antagonize the spasm of smooth muscle resulting from the application of barium chloride, the action of histamine on the gut and bronchioles, and the effects of epinephrine on kidney vessels. In addition they state that the drug will relieve all types of pain and in cats will abolish the cough reflex. From these findings they recommend the compound to be useful as a spasmolytic and analgesic agent. Recently this work has been repeated and extended by Duguid and Heathcote (2) who also conclude that it may be found effective as an antispasmodic drug. There are numerous reports in the German medical literature (3) ascribing beneficial effects from the use of Demerol (Dolantin) in a wide variety of pathological conditions. It has been used as an analgesic for the relief of both traumatic and congestive pain resulting from many causes. It has also been recommended as a spasmolytic agent in the treatment of spastic conditions of almost all types of smooth muscle. Although the chemical is reported to be effective in cases where there is pain only, it is claimed to be most effective where the pain is associated with spasm of smooth muscles, as in renal and biliary colic. Undesirable side effects as dizziness, euphoria, nausea and vomiting have been noted following the administration of therapeutic doses and four cases of addiction have been reported (4).

<sup>1</sup> This research was supported by a grant from the Alba Pharmaceutical Company, Incorporated.



In view of the drug's clinical use in Europe and its probable use in other countries, and the fact that it has been inadequately investigated on experimental animals, it was thought essential to carry out a thorough pharmacological and toxicological study of its properties.

#### EXPERIMENTAL PROCEDURES AND RESULTS

A description of the technique employed will be included in the introduction to each group of experiments. The doses usually employed in the experiments on intact animals were comparable, on a weight basis, to those used for clinical work in humans.

#### *Acute toxicity*

The visible responses of mice to toxic doses of Demerol are increased general activity, increased irritability, S-shaped tail reaction similar to that observed in mice with morphine poisoning, clonic convulsions, marked dyspnea and cyanosis, and in some animals death due to failure of respiration. Rabbits likewise respond to toxic doses of the drug by increased excitability, clonic convulsions, opisthotonos, dyspnea, Cheyne-Stokes respiration and in some animals death due to respiratory failure. Eisleb and Schaumann (1) report the minimum lethal dose of Demerol for mice as 150 mgm. per kilogram subcutaneously and 60 mgm. per kilogram intravenously, for rabbits 30 mgm. per kilogram intravenously and 700 mgm. per kilogram orally.

We have determined the acute toxicity of Demerol by intraperitoneal and oral administration in white mice weighing 15 to 25 grams; by intraperitoneal injection into albino rats weighing 100 to 175 grams; and by intravenous injection (at 50 mgm. per minute) into rabbits weighing 1.3 to 2.4 kgm. (av. = 1.7) and 2.3 to 4.2 kgm. (av. = 3.1).<sup>2</sup> The drug was administered as an aqueous solution, the concentration being adjusted to give a convenient volume for each type of injection.

On the basis of our observations we found the LD<sub>50</sub> of Demerol to be as follows:

ANIMAL	ROUTE OF ADMINISTRATION	LD <sub>50</sub> mgm. per kgm.
White mice. ....	Intraperitoneally	147
	Orally	221
Albino rats .....	Intraperitoneally	93
Rabbits (average weight 1.7 kgm.)....	Intravenously	32
Rabbits (average weight 3.1 kgm.)....	Intravenously	20

<sup>2</sup> The overlapping of the weight ranges is coincidental. Only one or two animals were used in each series in the overlapping range and the two groups of experiments were carried out on different occasions.



When the drug is given intravenously the weight of the animal as well as the rate of injection appears to be of great importance. Although the LD<sub>50</sub> for large rabbits was found to be 20 mgm. per kilogram when given at the rate of 50 mgm. per minute, a dose of 30 mgm. per kilogram killed only 1 of 6 animals when injected at the rate of 16 mgm. per minute.

Autopsies were performed on a large number of the rabbits used for acute toxicity determinations and in each animal the heart was found markedly dilated and the lungs contained many fine petechial hemorrhages.

### *Chronic toxicity*

Experiments on the chronic toxicity of Demerol were carried out by oral administration of an aqueous solution of the drug to white mice and by feeding capsules containing the compound to dogs.

A group of 10 mice was given a 0.05 per cent solution of Demerol as its sole source of fluid for a period of 25 days. During this time the average consumption was 3.6 cc. per day per mouse, which corresponds to 90 mgm. per kilogram per day per mouse. None of these animals showed any ill effects from this treatment.

Three dogs were given 100 mgm. per kilogram of Demerol in capsules wrapped in meat once daily. Within an hour after taking the second and each subsequent dose every animal developed some of the following symptoms: hyperirritability, unsteady gait, mydriasis, spastic extension of all extremities with loss of ability to stand, rapid shallow respiration, clonic convulsions precipitated either by noise or touch. Most of the earlier symptoms passed off within 24 hours and additional doses were administered. One animal died following the third dose, the other two survived six doses.

Complete blood counts were made before the start of the experiment and once weekly thereafter on four additional dogs to determine the possible effects of repeated administrations of Demerol on the blood picture. These animals were given 50 mgm. per kilogram by mouth once daily, six days a week, for five weeks. During this time the only observable gross findings were profuse salivation with some nausea—one animal vomited on a few occasions. All animals resisted the administration of the capsules after the first week, thus indicating some distaste for the treatment. At the end of the five weeks the dose was increased to 100 mgm. per kilogram per day and the experiment continued on the same schedule. All animals then developed the same symptoms found in the previous set of dogs. One female died after four doses at the higher level and one male died after the eighth. Both of these showed extensive hemorrhages into practically all tissues with free blood in the body cavities. The other two animals survived a total of 12 doses at the higher level and the experiment was discontinued. At no time during the entire 7 weeks was the blood picture of any animal significantly different from the control.



### *Respiration and blood pressure*

Another series of experiments was carried out on dogs under light ether anesthesia to determine the effects of intravenous injections of Demerol on the respiratory and vascular systems. Respiration was studied by means of a pneumograph attached to a recording tambour. The changes in blood pressure were recorded with a mercury manometer from a cannula placed in one carotid artery using heparin as the anticoagulant.

The intravenous injection of as little as 2 mgm. of Demerol per kilogram caused a sudden decrease in the depth and rate of respiration which soon returned to the control level (fig. 4). Repetition of these doses led to irregularity of rhythm and ultimately to cessation of respiration in the expiratory phase. Larger doses of the drug injected rapidly caused complete apnea and the animal might die without breathing again.

The drug was injected intravenously in doses of 0.25 to 10 mgm. per kilogram 134 times in 28 dogs. In 26 of the animals a sudden fall in blood pressure invariably followed the injection of the drug, and in some cases this fall amounted to as much as 140 mm. of mercury. The intensity of the effect appeared to be proportional to the dose and to the speed of the injection. Usually, though not always, the degree of fall seemed to decrease with repeated injections of a given dose. In 2 of the 28 dogs the initial injection of the drug caused a preliminary slight fall followed by a prolonged rise in blood pressure of as much as 30 mm. of mercury. All subsequent injections in these two animals showed a progressive increase in the depth of the early fall and a corresponding decrease in the height of the later rise in blood pressure.

In figure 1 can be seen the usual change in the blood pressure following the intravenous injection of Demerol. Five milligrams per kilogram of the drug were injected intravenously at 1 and the blood pressure fell from 108 to 67 mm. of mercury.

### *Skin*

Immediately after the injection of the drug there was obvious cutaneous vaso-dilatation, manifested by extreme reddening of the skin and thickening of the lips and ears.

### *Plethysmograph experiments*

Studies of the changes in the volume of the extremity, spleen, kidney, and intestine were made in an attempt to determine the cause of the fall in blood pressure. Twelve dogs were used in this investigation. Under surgical ether anesthesia oncometers were placed on one or more of the organs and connected to modified Brodie bellows for recording the volume changes. In a number of experiments a small rubber balloon was placed within the lumen of the segment of intestine which was enclosed in an oncometer. This



balloon was connected to a water reservoir which in turn was attached to a modified Brodie bellows. The pressure within this system did not exceed 15 cm. of water. This balloon recorded the activity of the segment of gut. Blood pressure readings were always made simultaneously with these other observations. The changes in volume of these organs following intravenous administration of Demerol can be seen in figures 1 and 2.

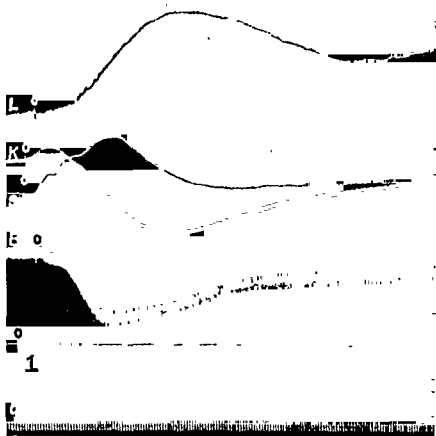


FIG. 1 Dog 13 kgm ♀. Ether anesthesia. Bottom record the time in intervals of 6 seconds and zero blood pressure and above it the time and speed of injection of the drug and 50 mm mercury pressure. *L*, volume record of rear extremity; *K*, kidney volume; *S*, volume of spleen, and *B*, the arterial blood pressure with mercury manom-

### *Limb*

As seen in figure 1, *L*, the extremity responded to the drug by an increase in volume. In this particular experiment the left hind leg was used and the left sciatic and femoral nerves had been sectioned previous to the injection. Similar changes in volume were found in all of the animals studied irrespective of the integrity of the nerve supply to the leg. The action appears to be definitely peripheral and not of central origin.



### Spleen

In our experiments the spleen (figs. 1, *S* and 2, *S*) always responded to the injection of the drug by a marked increase in size. The organ usually returned to normal within 15 minutes as can be seen in the figures. In a few animals the increase in volume of the spleen was followed by a decrease. In these instances there was always an extensive and prolonged fall in blood pressure. We believe that this decrease in size of the organ is not a drug effect, but is either a compensatory phenomenon (contraction of the smooth muscle in the organ) or a drainage of the blood away from the organ into other vascular channels. In a few experiments epinephrine was injected intravenously to decrease the volume of the spleen. In these the injection of Demerol intra-

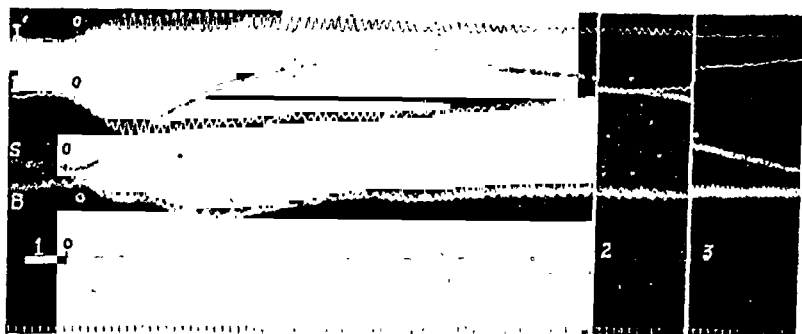


FIG. 2. Reduced to  $\frac{2}{3}$  original size. Dog 17 kgm. ♂. Ether anesthesia. Bottom record is the time marked in intervals of 6 seconds and zero blood pressure and the line above it indicates the time and speed of injection of the drugs and 50 mm. mercury pressure. *I'* is a record taken with a rubber balloon in the lumen of the segment of gut writing the volume record *I*. *S*, volume of spleen, and *B* the blood pressure with a mercury manometer. Upward movement of levers *I* and *S* indicates increases in volume and in *I'* increase in general tonus and contraction of the gut. For purposes of publication  $2\frac{1}{2}$  minutes of the record are omitted between 1 and 2 and  $3\frac{1}{2}$  minutes between 2 and 3. The *O* indicates simultaneous points of all the writing levers. At 1, 2 mgm. per kilogram of demerol injected intravenously.

venously quickly caused an increase in the volume of the organ even though a fall in blood pressure occurred.

### Kidney

Only a few experiments were performed in which the kidney volume was recorded. In these the organ showed a small and often insignificant increase in volume followed by an extensive decrease (fig. 1, *K*). The decrease in size of the organ parallels the fall in blood pressure and we believe it to be a passive phenomenon, the draining of the blood out of the organ.

### Intestine

The volume of the intestine in our experiments always decreased (fig. 2, *I*). Whether or not this is due to constriction of the blood vessels in the organ we



are unable to say. However, we are inclined to believe that the decrease is the result of the contractions of the intestinal smooth muscle, thus forcing the blood out of the organ and decreasing its size. This change in activity of the gut in response to Demerol is supported by our observations on the intact intestine in unanesthetized dogs (fig. 5). In figure 2, *I'* it will be noted that the general tonus as well as activity of the gut is increased during the period of decreased volume. It will also be noted that as the lever in *I'* goes up, showing a contraction of the gut, the lever in *I* recording the volume of the organ goes down, indicating a corresponding decrease in volume. This itself could explain the decreased volume of the intestine independent of direct vascular effects.

#### *Perfused blood vessels*

The Laewen-Trendelenburg (5) method of perfusion was employed in this investigation. The frog was pithed, the heart removed, and the animal

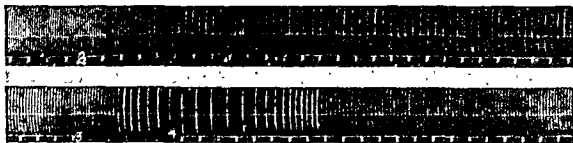


Fig. 3 Frog, *Rana pipiens*. Pithed. Perfusion flow of Ringer's solution in drops. 1. Perfusion flow of Ringer's solution in drops. 2. Epinephrine hydrochloride 0.2 cc. of into the perfusate. 3. A second injection of epinephrine. 4. Demerol, 1 cc of a 1 per cent solution injected into the perfusate.

perfused with Ringer's solution through the left aorta. The perfusion rate was recorded by permitting the fluid leaving the animal to drop on a receiving tambour which was connected to a recording tambour. The drug was injected into the perfusate through a hole in a glass tube covered by a rubber membrane.

The effect of Demerol alone on this animal preparation was inconsistent. In some instances a decreased flow was observed and in others a definite increase in the perfusion rate was noted. If, however, the rate of flow had been slowed by the injection either of 0.2 cc. of a 1:100,000 solution of epinephrine (adrenalin chloride) or of 0.3 cc. of a 1:50 solution of barium chloride, then 1.0 cc. of a 1 per cent Demerol solution quickly restored the perfusion flow approximately to normal. If the epinephrine and Demerol were mixed in a syringe and then injected, no change in the rate of perfusion was seen in our experiments.

In figure 3 can be seen the antagonism of this drug to epinephrine. In this



figure at 2, 0.2 cc. of a 1:100,000 solution of epinephrine were injected into the perfusate. The number of drops leaving the animal decreased from 32 to 9 per minute. Fifteen minutes after the injection the rate per minute, 24 drops, was still below the control. A similar injection was made at 3 where the perfusion rate decreased from 24 to 4 drops per minute and approximately one minute after this injection of epinephrine, at 4, 1.0 cc. of a 1 per cent solution of Demerol was injected into the perfusate with a resultant increase in the rate to 24 drops per minute within 5 minutes. As seen in this figure, epinephrine was twice as active on its second injection (due to slower rate of perfusion) nevertheless the previous control rate was restored in 5 minutes by Demerol. This was not accomplished within 15 minutes by perfusion alone.

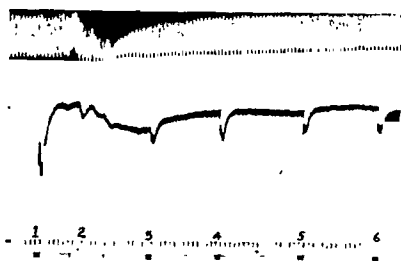


FIG. 4. Dog 8 kgm. ♂. Ether anesthesia. Top record is that of respiration and below it the blood pressure with a mercury manometer. Bottom record the duration of stimulation of the vagus nerve trunk and point and duration of the injection of demerol and above it the time in intervals of 15 seconds and zero blood pressure. Up-stroke in respiration record is inspiration. 1. Vagus nerve stimulated while the animal was under light ether anesthesia. 2. Injection of 10 mgm. per kilogram of demerol. 3, 4, 5 and 6. Vagus nerve stimulated as in 1.

#### *Vagus nerve*

The effect of the drug upon the irritability of the cardiac vagus nerves was studied in 11 dogs under light ether anesthesia. In each instance the right vagus nerve was exposed, severed, and its distal end prepared for stimulation with platinum electrodes connected to a Harvard inductorium. A tetanizing current was used. The fall in arterial blood pressure was used as the criterion of vagus irritability. A control vagus nerve effect was first established, following which Demerol in doses of either 5.0 or 10.0 mgm. per kilogram was injected intravenously. In 15 of the 20 injections made, definite decreases in the irritability of the right vagus nerve were noted. In the remaining 5 experiments the change in blood pressure upon vagus nerve excitation before and after the injection of the drug was so slight that it was regarded as negative.

In figure 4 can be seen the effects of right vagus nerve stimulation on arterial blood pressure before and after the injection of Demerol. The injec-



tion of the drug was followed by decreased respiration and a slight fall in blood pressure. Some time after the injection of the drug, stimulation of the vagus nerve continued to give a fall in blood pressure less than the control value even though the general arterial blood pressure had returned to its control level.

Nineteen experiments were performed on frogs and 15 on terrapins in which the effects of Demerol on the vagus nerve were studied (6). Exposure of the heart to dilutions of 1:10,000 to 1:50,000 caused marked depression and in most cases complete paralysis of the vagal nerve endings. In these experiments the heart failed to respond to stimulation of the right vagus nerve trunk or of the crescent.

### *Bronchial muscle*

Excised guinea-pig lungs were perfused according to the method proposed by Sollmann and von Oettingen (7). The perfusion fluid used was that proposed by Tainter, Pedden, and James (8). The pressure was kept constant throughout each experiment. The temperature of the perfusate as well as that of the air surrounding the lungs was kept at 38.5°C. by means of a large water bath. The change in perfusion rate was recorded by means of a tambour which indicated the bubbles of air entering the large reservoir (9).

As far as we could determine, normal bronchial muscle will be relaxed by Demerol only if it is in a state of partial contraction. In our experiments, after the muscles are once relaxed by Demerol neither it nor epinephrine has any further effect. These findings are supported by the fact that if the bronchial muscles are contracted in response to histamine acid phosphate, Demerol, like epinephrine, will relax them. However, the action of Demerol in this respect is less than that of epinephrine.

### *Stomach and pylorus*

The animals used in this research were the same as those previously employed by one of us (10). The method of recording the changes in action of these organs was described in that report and need not be repeated here. Fifteen experiments were performed on 6 different dogs. The dose varied from 0.5 to 2.0 mgm. per kilogram and was given by slow intravenous injection.

Eleven of the 15 injections were followed by marked temporary central nervous system excitement and 2 of the experiments had to be discontinued because of this. In the 13 completed experiments no change in the activity of the stomach was observed in 6, stimulation (more frequent contractions; increased amplitude of contractions; or increased general tonus) in 6, and increased followed by decreased activity in one. The results on the pylorus, however, were somewhat more uniform. In only 2 experiments was there no effect and in one there was stimulation followed by depression. Following



the remaining 10 injections some form of increased activity was noted. It can be stated definitely that this drug has no spasmolytic action on the stomach or pylorus in the unanesthetized dog.

### *Intact intestine*

These experiments were performed on trained, unanesthetized dogs which had either Thiry-Vella loops of the ileum or permanent duodenal fistulae. The necessary operative procedures had been carried out previously under surgical ether anesthesia and the wounds were completely healed before these experiments were begun. A rubber balloon was inserted into the lumen of the gut and connected to a pressure bottle containing water. This bottle was connected to a modified Brodie bellows which recorded the changes in activity and tonus on a smoked kymograph drum. The pressure within the balloon was approximately 15 cm. of water in each experiment. After sufficient time had elapsed for the intestine to become adjusted to this experimental procedure, as shown by a steady record, 0.5 to 2.0 mgm. per kilogram of a freshly prepared solution of Demerol were injected slowly into the saphenous parva vein. One week was permitted to elapse between successive experiments on the same animal.

Because of the uniformity of results only twelve experiments were performed using three dogs with Thiry-Vella loops and two with duodenal fistulae. An increase in the general tonus of the gut was observed following every injection. Borborygmus was usually present. In most instances Demerol also increased the peristaltic activity and the force of the rhythmic contractions of the intestine. During the period of increased activity the animals frequently gave evidences of excitement which we attributed to the severe intestinal contractions. In only one instance did the general tonus (after a prolonged elevation) fall slightly below the control level.

Figure 5 is presented as being fairly typical of all the experiments performed with the drug on the intact intestine of the nonanesthetized dog. In this figure at 1, between the arrows  $\downarrow \uparrow$ , 2.0 mgm. per kilogram of Demerol were injected intravenously into a 10.4 kilogram dog with a balloon in a Thiry-Vella loop. There resulted a sudden increase in the general tonus of the gut accompanied by powerful peristaltic contractions. This increased activity lasted for more than an hour.

Since Demerol has been recommended clinically as a spasmolytic agent it was thought essential to investigate the influence of the drug on contracted intestine. It has been shown that the intravenous injection of morphine sulphate will cause a spasm of the gut which can be partially antagonized by atropine sulphate (11) and by sodium phenobarbital (12). Morphine sulphate was, therefore, injected intravenously in doses of 1.0 mgm. per kilogram in 5 of these animals. During the increase in general tonus, which was observed in each instance, 2.0 mgm. per kilogram of Demerol were



injected intravenously. As far as could be determined, this latter injection had no significant effect upon the morphine response.

### *Isolated intestine*

Observations were made by simultaneously suspending a segment of duodenum and one of ileum in a bath of Tyrode's solution (pH = 7.6) kept at 38.5°C. (13). Aqueous solutions of the various drugs were added to the bath in amounts sufficient to provide the desired final concentrations. A total of 153 observations, made on 42 different pairs of segments taken from 11 animals (rabbits and cats), showed neither a species difference nor a difference between the responses of the duodenum and ileum.

Demerol was used in dilutions varying from 1:1,000,000 to 1:5,000 and some depressions and some stimulations were observed with all dilutions used. The action of the drug under these circumstances seems to depend

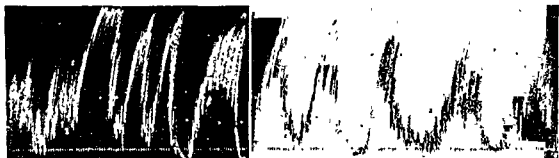


FIG 5. Reduced to  $\frac{1}{3}$  original size. Dog 10.4 kgm. ♀. Unanesthetized with Thirly-Vella fistula of ileum. Top record is of intestine and bottom record is the time in intervals of 15 seconds. Upstroke indicates contraction of muscle and increased tonus. At 1, 2.0 mgm. per kilogram of demerol were injected intravenously. For publication purposes 15 minutes of the control record have been omitted and 5 minutes between records A and B.

upon the state of the tissue at the particular time the compound is added to the bath. If the muscular tonus is low, the drug tends to increase it, but if the tonus is high the reverse usually takes place. Likewise, if the amplitude of the rhythmic contractions is small the drug tends to increase it; if large the reverse is again noted. The action on this tissue is, therefore, quite unpredictable and unreliable. The effects of the drug, when they do appear, are readily reversible and the return of the segments to the control state after washing with fresh Tyrode's solution is fairly prompt.

If the segments of intestine are relaxed by the addition of epinephrine to the bath (1:5,000,000 dilution) then Demerol in 1:10,000 dilution will usually quickly antagonize the epinephrine effect. If the same segments are contracted by the addition of either acetylcholine (1:5,000,000 dilution) or barium chloride (1:10,000 dilution) to the bath in which they are contracting, Demerol now added to the bath in dilutions of 1:10,000 to 1:5,000 antagonizes, either partially or completely, these effects.



*Intact uterus*

Dogs under light ether anesthesia were used and the uterine contractions recorded by the method described by Barbour (14). Blood pressure changes were recorded simultaneously from the carotid artery. Demerol in doses of 2.0 or 5.0 mgm. per kilogram was injected into the femoral vein.

In our experiments it was found that whenever Demerol had an effect it was that of stimulation and not depression of the uterus. Figure 6 is a

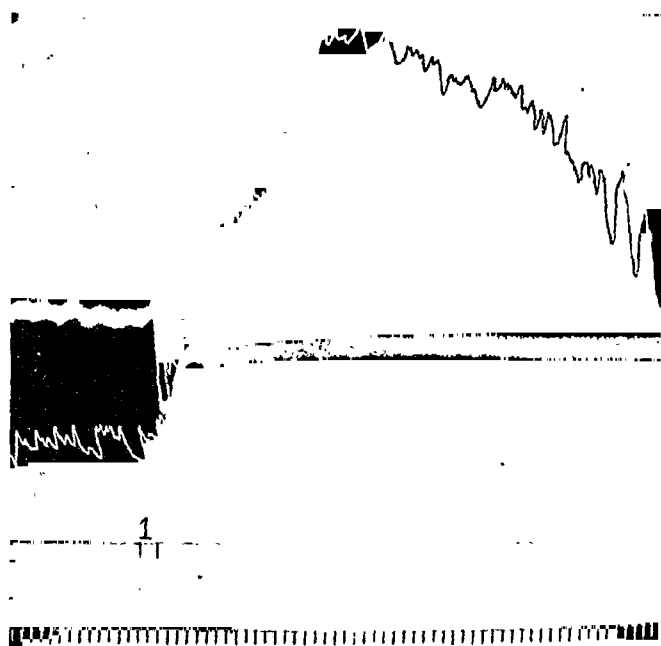


FIG. 6. Dog 9.1 kgm. ♀. Ether anesthesia. Top record the blood pressure in mm. of mercury and below it that of the intact uterus. Bottom record the time in intervals of 15 seconds and zero blood pressure and above it the point of injection of the drug and 50 mm. mercury pressure. Thirty minutes of control record omitted. Upstroke in uterine record indicates contraction. At 1, 5.0 mgm. per kilogram of demerol were injected intravenously.

typical record. At 1 in this figure, 5.0 mgm. per kilogram of the chemical were injected intravenously. There resulted a slight fall in blood pressure and simultaneously a marked contraction of the intact uterus. Upon comparing this action with that of papaverine, one might infer that the fall in arterial blood pressure was not instrumental in producing the contraction of the uterus. Both papaverine hydrochloride (1.0 mgm. per kilogram) and epinephrine (0.006 or 0.02 mgm. per kilogram) injected intravenously caused marked relaxations of the uterus. Pituitrin "S" in doses of 0.02 and 0.025



cc. per kilogram caused a much greater and more sustained contraction of the organ than did Demerol.

Pregnancy may not be a contraindication for the use of this drug. One of our dogs used for chronic toxicity studies gave birth to a litter of perfectly normal pups although she had received 50 mgm. per kilogram of the chemical each day for 3 weeks.

### *Excised uterus*

These experiments were performed on segments of non-pregnant uteri taken from rabbits and cats. Two segments (one from each horn) were observed simultaneously and a total of 11 such pairs was used. The experiments were carried out in the usual manner by suspending the segments in aerated Locke's solution adjusted to pH 7.6 and kept at 38.2°C (15). Twenty-three experiments were performed in which Demerol alone was added to the bath. In these, uterine segments which were not contracting usually



FIG. 7. Reduced to  $\frac{1}{4}$  original size. Excised segments of rabbit uterus. Locke's pH 7.6, temperature 38.2°C and aerated. Time in intervals indicates contraction of muscle and increased tonus 1 (1.500,000). 2. Demerol added to bath (1:10,000) 3. Bath replaced by fresh Locke's solution. 4 Pituitrin "S" added to bath (0.4 cc. to 100 cc. Locke's solution). 5. Demerol added to bath (1.10,000) 6 Replaced bath with fresh Locke's solution.

contracted and this procedure often initiated rhythmic contractions which continued even after the drug was removed. When the chemical was added in the presence of active segments the general tonus usually increased somewhat. In 4 of the 23 experiments the drug decreased either the force of the rhythmic contractions or the general tonus.

Epinephrine was added to baths containing rabbit or cat uteri 18 and 5 times respectively. In the experiments on rabbit uteri the addition of Demerol (1:10,000 dilution) either partially or completely antagonized the contraction caused by the epinephrine (fig. 7 at 2). In two of these cases only one of the pair of segments responded by relaxation. In the experiments on cat uteri Demerol caused contraction of the relaxed uterus 4 times and had no effect upon the epinephrine action once. In the only experiment in which histamine acid phosphate was used the drug antagonized its contracting effect.

Pituitrin and barium chloride were added to the bath 8 and 5 times respectively and in these Demerol (1:10,000 dilution) caused partial to complete



relaxation of the contracted uterine segments. In the remaining experiments the drug apparently had no effect (fig. 7 at 5). A comparison of the relaxing properties of this drug and those of *ortal* sodium was made and it was found that the latter is far superior to the former in equal concentration. In the literature one reads of the superiority of this drug over papaverine as a spasmolytic agent. In our hands papaverine always acted as a depressant drug on the uterus whereas Demerol had the opposite effect.

### *Urinary tract*

Experiments were carried out on dogs under light ether anesthesia. The ureters were isolated, cannulated, and tambours arranged to record the combined flow from the two kidneys in drops. The bladder was cannulated either through the dome or through the urethra and filled with fluid under a very slight positive pressure; a tambour was also arranged to record changes in the bladder volume. The drugs were injected into the femoral vein and the blood pressure was recorded from the carotid artery. The dose of Demerol varied from 1.0 to 5.0 mgm. per kilogram. A total of eight injections was made.

A slight contraction of the bladder lasting for about one and a half minutes was seen on 5 occasions while the remaining experiments showed no changes in volume. As a check on the method epinephrine was injected intravenously. In these experiments typical epinephrine responses were seen.

The effects of Demerol on the urine secretion are uncertain. If there is an action it is that of decreased secretion. This may be due to the decreased arterial blood pressure.

### SUMMARY AND CONCLUSIONS

The LD<sub>50</sub>, per kilogram, of Demerol is: for white mice, 147 mgm. intraperitoneally and 221 mgm. orally; for albino rats, 93 mgm. intraperitoneally; and for rabbits weighing 1.3 to 2.4 kilograms, 32 mgm. and those weighing 2.3 to 4.2 kilograms, 20 mgm. intravenously. The drug does not appear to alter the blood picture of dogs when given in large doses over a considerable period of time.

Doses as low as 0.25 mgm. per kilogram given intravenously usually produce a fall in blood pressure, due primarily to peripheral vasodilatation. The intensity of the effect is dependent upon the dose and the rate of injection of the drug. The respirations are temporarily decreased in depth and frequency. Repetition of small doses at intervals of about 15 minutes ultimately leads to death by respiratory failure. The spleen and extremities increase in volume while the intestine shows a decrease in size. The decrease in size of the intestine is due to the powerful contractions of the intestinal smooth muscle. After a preliminary increase the kidney decreases in size. The



decrease is passive, due to the fall in blood pressure. Depression of the irritability of the cardiac vagus nerve is usually observable and this action is probably on the postganglionic vagal nerve endings.

The action of Demerol on excised smooth muscle (intestine, bronchus, uterus, blood vessel) is unpredictable. If the muscle is relaxed the drug usually causes some contraction, while if it is contracted, relaxation is often seen. The effects of epinephrine hydrochloride are usually antagonized and the actions of barium chloride, histamine acid phosphate, and pituitrin "S" are usually partially if not completely counteracted. The spasmolytic potency of Demerol on isolated tissues is less than that of the more active barbiturates (oral and evipal) and papaverine hydrochloride.

On intact smooth muscle (stomach, pyloric sphincter, small intestine, uterus, and urinary bladder) the drug shows no promise of value as a spasmolytic agent; contractions of the muscle occur. Any value that the drug may have in relieving intestinal colic must, therefore, be attributed to its analgesic potency.

From our experiments on intact and excised uteri it seems obvious that this chemical is of no value as a uterine sedative. The stimulant action, which is the most common finding, is much less potent than that of pituitrin. Pending complete and careful clinical investigation extreme care should be exercised in administering this drug to pregnant women.

The relief experienced by patients suffering with urinary bladder tenesmus must be due to the analgesic rather than the spasmolytic action of the drug.

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# THE EFFECT OF CALCIUM AND STRONTIUM SALTS ON THE ACTION OF SODIUM PENTOBARBITAL

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Received for publication August 1, 1941

It was observed previously in rats that sodium pentobarbital in anesthetic doses reduced the deaths from strontium acetate by vein but did not reduce the toxicity of calcium acetate under similar circumstances (1). Apparently conflicting with these data are the results of Carratala (2), who reported that veronal, dial and somnifen reduced the toxicity of calcium chloride by vein in dogs and rabbits. Although the responsibility for this apparent discrepancy probably rests on a difference in the species, it is interesting to note that the mechanism of death produced by calcium salts is about the same in the three species (1, 2).

When our studies were made, it seemed that those rats receiving strontium or calcium acetates which recovered were under the influence of the anesthetic longer than similar animals which received the same dose of sodium pentobarbital alone. No reports of the effect of strontium on the duration of action of the commonly used general anesthetics or sedatives were found in the literature. The local anesthetics have been studied and it has been reported that strontium and calcium chlorides increased the anesthetic action of cocaine, stovaine, and procaine (3, 4). Also calcium chloride has been reported to inhibit magnesium sulfate narcosis while strontium chloride stimulated it (5). These facts aroused interest in further studies on the relationship between strontium and calcium and sodium pentobarbital in regard to the duration of anesthetic action and toxicity.

## METHODS

The sodium pentobarbital was from a commercial preparation containing 10 per cent alcohol and 65 mgm. per cubic centimeter of sodium pentobarbital. The strontium acetate was given as a freshly prepared 5 per cent solution, the calcium acetate was 4.11 per cent, equimolecular with the strontium acetate solution. Not less than twenty animals were used for each determination. The rats for each study were adults matched for sex and weight. Intraperitoneal injections were given by accurately graduated syringes. The intravenous injections were given from a 5 cc. burette. The acetate immediately preceded the sodium pentobarbital when given by intraperitoneal injection. The injection of acetate immediately followed the sodium pentobarbital when given by intravenous injection. Separate syringes or burettes and needles were used for each drug throughout. When two drugs were given both were given by the same route.



For points of reference on the duration of anesthesia the loss and regaining of the righting reflex were used. The rats were laid on their backs on a smooth wooden table top immediately after the administration of the sodium pentobarbital. If the rat righted himself this procedure was repeated at 2-minute intervals until the rat no longer turned on to his feet. This time was noted and recorded as the time of the loss of righting reflex. The rat then lay undisturbed on his back until he spontaneously rolled over and put his weight on his front paws. This was taken as the time of regaining of the righting reflex. The difference between these two times is referred to in the tables as the duration of anesthesia. Toxicity studies were based entirely on death of the animals.

### RESULTS AND DISCUSSION

With intraperitoneal injection both calcium and strontium acetates increased the duration of anesthesia significantly and to about the same degree

TABLE 1  
*Duration of anesthesia*

SODIUM PENTOBARBI- TAL	CALCIUM ACETATE	STRONTIUM ACETATE	NUMBER OF RATS	DURATION OF ANESTHESIA		S.E.M
				Range	Average	
Intraperitoneal						
mgm. per kgm.	mgm. per kgm.	mgm. per kgm.		minutes	minutes	
21	0	0	20	0-56	32	$\pm 3.1$
21	0	250	20	0-80	41	$\pm 3.9$
32	0	0	20	0-94	68	$\pm 5.0$
32	0	250	20	0-145	95	$\pm 7.4$
32	210	0	19*	0-149	100	$\pm 8.9$
Intravenous						
21	0	0	30	19-175	90	$\pm 6.8$
21	0	200	30	45-166	104	$\pm 0.97$
21	164	0	20	79-223	149	$\pm 9.1$

\* One rat died without recovering from the anesthetic and six others died later.

(table 1, figs. 1 and 2). The calcium acetate, however, killed seven of the twenty animals used. When the salts were given intravenously with sodium pentobarbital there was a questionable difference between sodium pentobarbital alone and sodium pentobarbital with strontium acetate, whereas calcium acetate produced a marked increase in the duration of anesthesia (table 1, fig. 3). In earlier studies the strontium ion was observed to be more easily washed out of the tissue than the calcium ion (6), and it is possible that this difference in diffusibility accounts for the fact that strontium did not prolong anesthesia to the degree which calcium did by the intravenous route.

That diffusibility was a factor in the toxicity after intraperitoneal injection seemed likely. Further studies on the effect on sodium pentobarbital on the toxicity of strontium and calcium are summarized in table 2. Again the toxic-



## INTRAPERITONEAL INJECTION

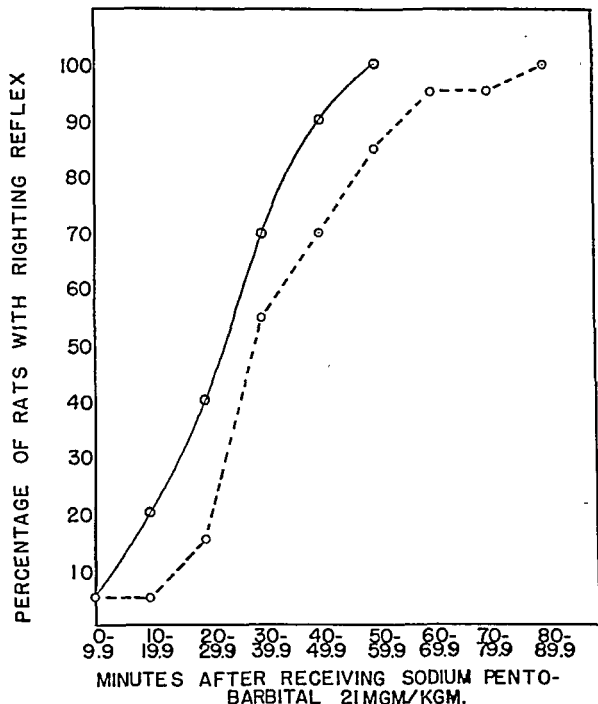
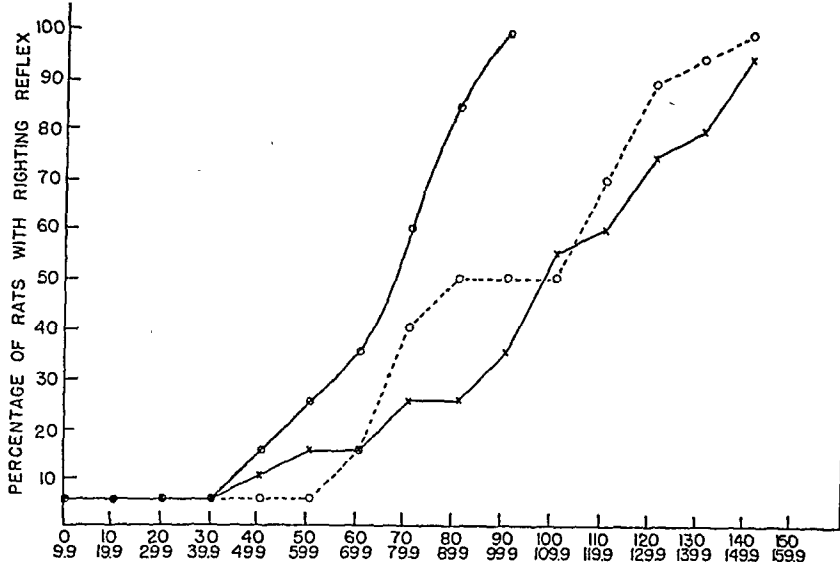


FIG. 1. DURATION OF ANESTHESIA AFTER INTRAPERITONEAL INJECTION OF SODIUM PENTOBARBITAL, 21 MGM./KGM.

○——, 20 rats received sodium pentobarbital only; ○——, 20 rats received sodium pentobarbital + Sr. acetate, 250 mgm./kgm.

ity of strontium by vein is shown to be decreased at 200 mgm. per kilogram as well as at the higher dosage level (1), whereas the toxicity of calcium was not appreciably changed. The most striking observation from this study seemed

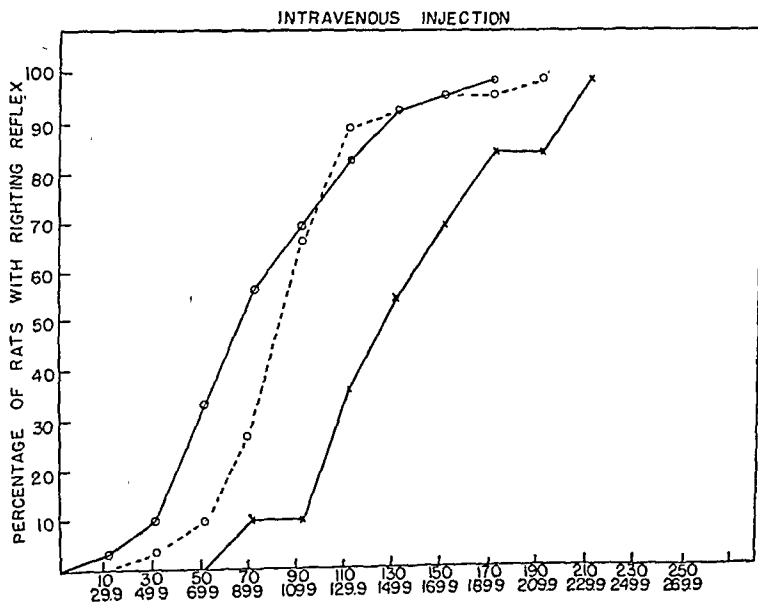




MINUTES AFTER INJECTION OF SODIUM PENTOBARBITAL 32 MGM./KGM.

FIG. 2. DURATION OF ANESTHESIA AFTER INTRAPERITONEAL INJECTION OF SODIUM PENTOBARBITAL, 32 MGM./KGM.

○——, 20 rats received sodium pentobarbital only; ○-----, 20 rats received sodium pentobarbital + Sr. acetate 250 mgm./kgm.; X——, 20 rats received sodium pentobarbital + Ca. acetate 210 mgm./kgm.



MINUTES AFTER INJECTION OF SODIUM PENTOBARBITAL 21 MGM./KGM.

FIG. 3. DURATION OF ANESTHESIA AFTER INTRAVENOUS INJECTION OF SODIUM PENTOBARBITAL, 21 MGM./KGM.

○——, 30 rats received sodium pentobarbital only; ○-----, 30 rats received sodium pentobarbital + Sr. acetate 200 mgm./kgm.; X——, 20 rats received sodium pentobarbital + Ca. acetate 210 mgm./kgm.



to be the change in toxicity with a change in the mode of administration. The strontium acetate is strikingly less toxic by intraperitoneal injection than by intravenous injection and the calcium acetate is slightly more toxic by intra-

TABLE 2

*The effect of sodium pentobarbital on calcium and strontium*

SODIUM PENTOBARBITAL	CALCIUM ACETATE	STRONTIUM ACETATE	NUMBER OF RATS	NUMBER DIED	PER CENT DIED	S.E.M.
Intraperitoneal						
mgm. per kgm.	mgm. per kgm.	mgm. per kgm.				
0	0	250	20	0	0	±7.9
0	210	0	40	19	47.5	
32	0	0	20	0	0	
32	0	250	20	0	0	
32	210	0	20	7	35.0	±10.7
Intravenous						
0*	0	250	20	9	45	±11.1
0*	210	0	20	5	25	±9.7
0*	0	200	20	8	40	±10.9
0*	164	0	20	2	10	±6.7
21	0	0	30	0	0	
21	0	200	30	0	0	
21	164	0	20	0	0	

\* Data taken from (1).

TABLE 3

*Hemoglobin after intraperitoneal injection*

TIME AFTER DRUG	CALCIUM ACETATE: ANIMALS DIED			CALCIUM ACETATE: ANIMALS LIVED			STRONTIUM ACETATE		
	Number of rats	Hemo- globin	S.E.-M	Number of rats	Hemo- globin	S.E.	Number of rats	Hemo- globin	S.E.-M
hours		grams per 100 cc.			grams per 100 cc.			grams per 100 cc.	
Control	11	13.6	±0.42	9	13.8	±0.09	20	13.8	±0.26
1	7	17.9		8	18.1	±0.56	15	15.8	±0.36
3	11	19.2	±0.34	9	19.2	±1.16	20	15.9	±0.41
6	9	19.6		9	17.5	±0.55	20	15.0	±0.26
9	9	19.5		9	17.1	±0.30	20	14.2	±0.30
24	1	18.0		9	14.7	±0.66	20	13.0	±0.31
48				9	13.2	±0.45	20	12.5	±0.28
72				9	12.6	±.84	20	12.5	±0.33

peritoneal injection. If the greater toxicity of calcium as compared with strontium acetate were due to the calcium ion being less diffusible than the strontium ion then the osmotic pressure of the calcium in the peritoneal cavity



would result in a dehydration of the tissues with a consequent concentration of erythrocytes in the blood. Table 3 and figure 4 show that there was a higher hemoglobin concentration in the blood of the rats which received calcium acetate than in those which received strontium acetate. Furthermore at 6

### INTRAPERITONEAL INJECTION

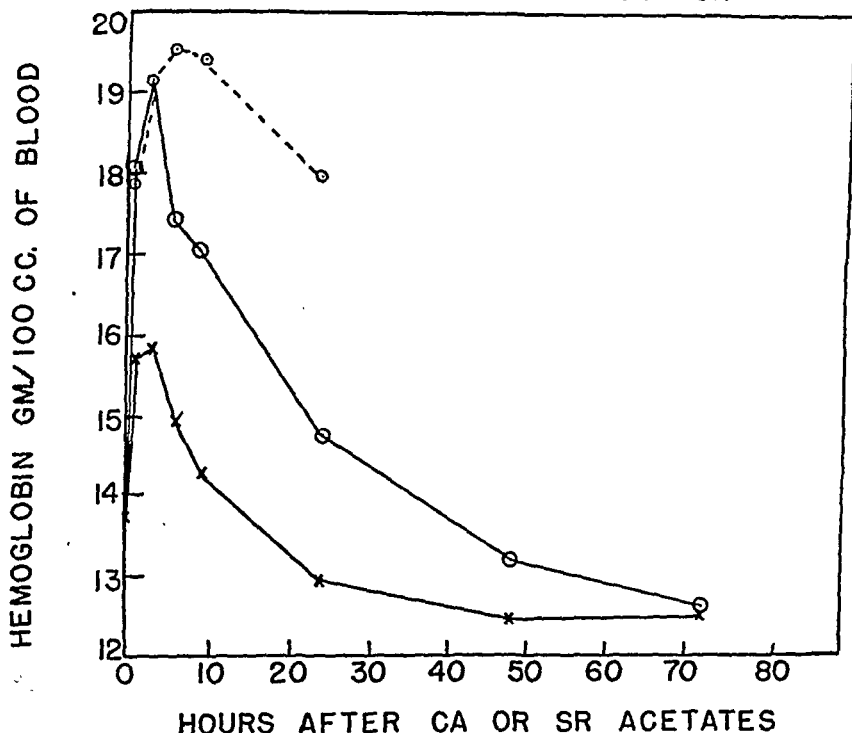


FIG. 4. HEMOGLOBIN CONCENTRATION AFTER INTRAPERITONEAL INJECTION OF CALCIUM AND STRONTIUM SALTS

X—, strontium acetate, 250 mgm./kgm.; O—, calcium acetate, 210 mgm./kgm. (rats survived); O—, calcium acetate, 210 mgm./kgm. (rats died).

and 9 hours after injection a markedly higher hemoglobin was observed in the rats which later died from calcium injection than in those which survived.

Since sodium pentobarbital reduced the toxicity of strontium acetate an experiment was designed to see if the reverse held true. A highly toxic dose of sodium pentobarbital (128 mgm. per kilogram) was tried with and without a non-toxic dose of strontium acetate (250 mgm. per kilogram). Both drugs were given separately by intraperitoneal injection. The results are shown in



figure 5. Strontium acetate decreased the survival time and in no way antidoted the sodium pentobarbital.

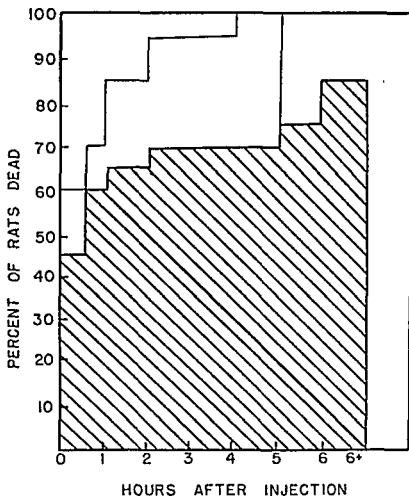


FIG. 5. DEATHS AFTER SODIUM PENTOBARBITAL, 128 MGm./KGm. BY INTRAPERITONEAL INJECTION

▨, sodium pentobarbital only; □, sodium pentobarbital + Sr. acetate, 250 mgm./kgm.

#### SUMMARY

1. Both calcium and strontium acetates increased the duration of sodium pentobarbital anesthesia when the salts were given in equimolecular quantities by intraperitoneal injection.

2. Calcium acetate increased the duration of sodium pentobarbital anesthesia when given by vein. Strontium acetate had very little if any effect.

3. Calcium acetate was more toxic to the rat by intraperitoneal injection than strontium acetate.

4. The greater toxicity of the calcium by intraperitoneal injection was shown to be associated with an increased hemoglobin concentration of the blood.

5. Sodium pentobarbital decreased the toxicity of strontium acetate but strontium acetate did not decrease the toxicity of sodium pentobarbital.



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# ON THE MODE OF ACTION OF THE SULFONAMIDES

## I. ACTION ON *ESCHERICHIA COLI*<sup>1</sup>

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Received for publication August 4, 1941

Believing that the mode of action of the sulfonamides, though basically the same in all species, may be modified by the metabolic differences among them, we have undertaken a systematic and quantitative study of two different metabolic types. In this and the succeeding paper we shall present results obtained with a strain of *E. coli*, to illustrate the situation in an organism possessing the highest degree of synthetic ability. Later, we shall describe comparable experiments on the more specialized *Hemophilus parainfluenzae*, which can serve for extended comparisons.

For purposes of future discussion, it will be convenient to adopt, at least tentatively, a theoretical approach to the problem. The work of Stamp (1) and of Green (2) which led to the discovery by Woods (3) that *p*-amino-benzoic acid antagonizes the sulfonamides provides a reasonable and definite orientation. The English school has postulated (3, 4) that this acid, or a closely related substance, is essential for the growth of susceptible cells, and that the enzyme reaction which involves it is subject to competitive inhibition by the sulfonamides. Most organisms, apparently, are able to effect its synthesis, but *Clostridium acetobutylicum* requires it as a vitamin (5).

Rather less definite ideas have been advanced by others. Levaditi (6) assumed the drugs to interfere with nitrogen metabolism. Lockwood, who discovered that peptones are antagonists, also believes that somehow nitrogen metabolism is affected (7). Fuller, Colebrook and Maxted (8), who independently discovered the action of peptone, proved that sulfonamide does not interfere with proteolysis, and believe that sulfanilamide "acts in media in which the streptococcus grows comparatively poorly and fails to act decisively in media in which it grows luxuriantly." Gay, Clark, Street and Miles (9) have argued for the necessity to consider "the sum total of factors that inhibit or favor the natural growth of the microorganism under the experimental conditions. . . ." McIntosh and Whitby (10) state that the "drugs in some way block the vital food-supply of the bacteria, . . . probably by neutralization of some metabolic function or enzymatic activity."

<sup>1</sup> Aided by a grant from the Duke University Research Council.



Of these views, we believe that of Woods to be the most advantageous. There are, however, a number of experimental facts and questions for which it does not seem adequate. Are we to assume, for example, that the antagonistic action of peptones, tissue extracts, and urine resides solely in their content of *p*-aminobenzoic acid? There is good evidence that this is not the case (8, 11). Do bacteria become resistant solely because of an enhanced capacity to synthesize *p*-aminobenzoic acid? Our own data (12) on the development of resistance to *Hemophilus* do not favor this. How shall we account for the fact that cozymase (13) and to some extent nicotinamide (14) are antagonists in certain species?

We suppose that many such difficulties can be resolved by an extension of the Woods formulation to include the behavior of reactions occurring, as of were, before and after the *p*-aminobenzoic acid step. And in dealing with our data we shall always make this attempt before adopting another point of view.

#### METHODS

The cultures were grown in tubes containing medium made up to a final volume of 7 or 8 cc. and incubated in a water bath at  $37 \pm .01^\circ$ . Inocula were obtained from actively growing cultures, whose density was determined photometrically, by diluting them appropriately with fresh medium.

*Organism.* *Escherichia coli*, no. 6522, American Type Culture Collection, was selected because it grew well on inorganic salts, ammonia, and glucose. The stock cultures were carried in medium SG or stored in vacuo after drying in the frozen state. This strain is well stabilized; organisms obtained in October 1938 were found to be quantitatively similar in behavior to those received in October 1940.

*SG medium.* Dissolve in 500 cc. of tap water plus 500 cc. of distilled water 4 grams of NaCl, 0.2 grams of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 grams of  $\text{KH}_2\text{PO}_4$ , 2 grams of  $(\text{NH}_4)_2\text{HPO}_4$  and 2 grams of glucose; bring to pH 7.2 with NaOH; filter and tube; autoclave for 15 minutes at 20 pounds. A precipitate forms which redissolves in a few hours.

Medium SG was employed because it required the maximal synthetic effort from the organisms. Glucose was chosen as the source of energy because growth was more rapid with it than with lactate or succinate. The addition of small amounts of bicarbonate did not accelerate growth. The maximum population reached in this medium is about  $8 \times 10^8$  organisms per cubic centimeter; at this point the pH has dropped to 6 to 5.3 depending upon the size of inoculum.

The organisms are sensitive to slight changes in this medium, and therefore each experiment was carried out with tubes from the same batch. The type of glass tube employed may also affect the growth rate. We found that Pyrex and new soft glass tubes gave consistent results, but old tubes of soft glass did not. The inconsistencies were not removed by washing the tubes with soap, calgonite or strong sulfuric acid-dichromate mixture. It was also found that autoclaving the tubes (filled with medium) at 20 pounds for less than 12 or more than 20 minutes might produce unfavorable results. Furthermore, old soft glass tubes which gave inconsistent results when autoclaved for 11 minutes, gave much better results when autoclaved for 15 minutes. Hence for quantitative experiments we tubed SG in Pyrex and autoclaved for 15 minutes at 20 pounds. These tubes were never filled with any other medium.

*PPFG medium.* Dissolve in 1000 cc. of distilled water 20 grams of proteose peptone



(Difco), 6 grams of NaCl, 2 grams of glucose and 0.4 grams of fumaric acid. Adjust the pH to 7.8 with NaOH. Filter and tube (7 cc.). Autoclave at 20 pounds for 12 minutes. This medium was employed because it requires a minimal synthetic effort from the organism. Growth is more rapid in it than in complete mixtures of the individual amino acids. Glucose is not necessary for growth, but greatly accelerates it. The maximum population reached is about  $1.5 \times 10^9$  organisms per cc., the final pH being 4.7. The duration of autoclaving and type of tube are not critical.

*Solid medium.* The medium used for pouring plates differed from PPFG in that it contained 2.2 per cent agar and 1.5 mgm. per cent p-aminobenzoic acid, but no fumarate. The pH was 7.4.

*Determination of bacterial population.* (1) Viable counts were usually performed by the method of plating according to the rules set forth by Wilson (15), or by the serial dilution technique in proteose peptone medium (16).

(2) Total counts were estimated by photometry in the Evelyn photoelectric colorimeter, using filter 520 M and growing the cultures in the usual soft glass photometer tubes or in selected  $22 \times 175$  mm. Pyrex tubes. The factor for converting turbidity readings into number of organisms was determined by growing fifteen cultures of *E. coli* in the presence and absence of sulfanilamide and comparing the optical density with direct counts in counting chambers. The factor of  $3.3 \times 10^8$  converted optical densities between 0.015 and 0.16 into organisms per cc. This factor was not changed by the presence of sulfanilamide. The total count from photometry in SG was generally 15 to 30 per cent more than the viable count as determined by plating.

*Assessment of bacterial growth rate.* The rate of increase in number of a homogeneous bacterial population depends upon the number of bacteria present and the rate of growth. In a suitable environment, the rate of growth will be constant; then the number of organisms,  $N$ , present at any time,  $T$ , after inoculating  $N_0$  into the medium will be

$$\log_e N = \log_e N_0 + KT \quad (1)$$

where  $K$  is the velocity constant of the rate of growth.<sup>3</sup> The numerical value of  $K$  is determined graphically by plotting  $\log_e N$  against  $T$  (we shall use minutes as the unit of time); a straight line whose slope is  $K$  should fit the points. Such data are shown in figures 3 and 6 for media PPFG and SG with and without sulfanilamide. In the absence of the drug there is a linear relationship (following a short latent period) between  $\log N$  and  $T$ , proving the rate of multiplication to be constant. In the presence of drug a curvilinear relationship is found, the slope decreasing with time. To calculate the slope at any point on such a curve, use is made of equation 2 which for convenience uses logarithms to the base 10.

$$K = \frac{2.3}{T_2 - T_1} \log \frac{N_2}{N_1} \quad (2)$$

$T_2$  is chosen as close to  $T_1$  as possible and the mean value of  $K$  for this interval is obtained.

The value of  $K$  was obtained by three methods.

(1) Viable count ( $K_v$ ). Plates were poured at suitable intervals from the culture. The data were plotted as in figure 3 and  $K$  calculated according to equation 2. In figure 3 the control  $K_v$  in SG equals 0.020. Roughly, this means that 2 per cent of the population divide each minute; precisely, it represents the instantaneous rate of multi-

<sup>3</sup> For a full discussion of this constant see Buchanan and Fulmer (16) who call it the growth constant.



plication. The control  $K$ , in PFG equals 0.040, and hence the rate of multiplication in the richer medium is twice that in the poorer one. The constants determined by this method will bear the subscript  $v$ .

(2) Mean value, photometrically ( $K_m$ ). A small inoculum usually between 100 and 500 organisms, is obtained by diluting an actively growing culture whose density is known from photometric estimation. The time is measured for this inoculum to reach the fixed end point of 132 million per cc.  $K$  is then calculated by substitution of these data into equation 2. Obviously, the value of  $K$  obtained is a mean for the entire period and therefore is designated by the subscript  $m$ .

(3) Serial dilutions, photometrically ( $K_{sd}$ ). In order to use photometry for the more exact determination of  $K$  as a function of time, a series of tubes is inoculated stepwise with large inocula so that the photometric endpoint (132 million per cc.) is reached after 2, 3, 4, 5, 6, and 7 divisions, respectively. The number of divisions is then plotted as a function of time, from which the rate of growth at any time or any number of divisions may be obtained, as follows. If  $D$  be the number of divisions which has occurred, then  $N = N_0 \times 2^D$ , and by the use of equation 2 it follows that  $K_{sd} = 0.69 D/T$ .

## RESULTS

We have measured the effect of the sulfonamides upon the velocity constant of growth of *E. coli* and also upon the respiration. The independent variables were: size of inoculum drug concentration, duration of exposure to drug, type of culture medium, and changes in the metabolic activity of the cells.

### *Size of inoculum*

$K_m$  is plotted as a function of the size of inoculum in figure 1 for medium PFG. In the absence of sulfonamide,  $K_m$  equalled 0.037 in this experiment and was constant though the inoculum varied a million fold. In the presence of  $10^{-3}$  M sulfanilamide,<sup>3</sup>  $K_m$  at first rapidly declined as the inoculum became smaller and then finally became constant. Similar changes in  $K_m$  with variation in size of inoculum were seen in the case of  $10^{-3}$  M sulfapyridine and sulfathiazole. With inocula smaller than 200,  $K_m$  was constant in all media.

In SG the results were similar but more variable. The variations in  $K_m$  with size of inoculum are plotted in figure 2 as percentages of the respective control  $K_m$  for each of four experiments. In the absence of drug, the control  $K_m$  was independent of the size of inoculum, though it varied from 0.014 to 0.018 in the different experiments. In the presence of sulfanilamide (and similarly in sulfapyridine, sulfathiazole and sulfadiazine)  $K_m$  was constant for inocula smaller than 15,000 organisms, but tended to increase as the inocula were made greater. This effect was more marked in the larger concentrations of sulfanilamide.

These results are confirmed and explained by an experiment using the viable count technic. The number of organisms growing from large (76,000

<sup>3</sup> We shall employ molar concentrations. For the various drugs,  $10^{-3}$  M is equivalent to mgm. per 100 cc. as follows: sulfanilamide, 17.2; sulfapyridine, 24.9; sulfathiazole, 25.5; and sulfadiazine, 25.



and 21,000) and small (380 and 125) inocula in media SG and PPFG, with and without sulfanilamide, is plotted against time in figure 3. It is apparent that size of inoculum (when this is below 76,000) has no effect on the result, and that six or seven hours of growth are necessary for the full development of the inhibition. Since very large inocula exhaust the medium and cease growing in less than six or seven hours, their use must lead to fallacious results because the full inhibition cannot develop. Hence in figures 1 and 2, as the size of inoculum decreased,  $K_m$  decreased and approached the limiting value

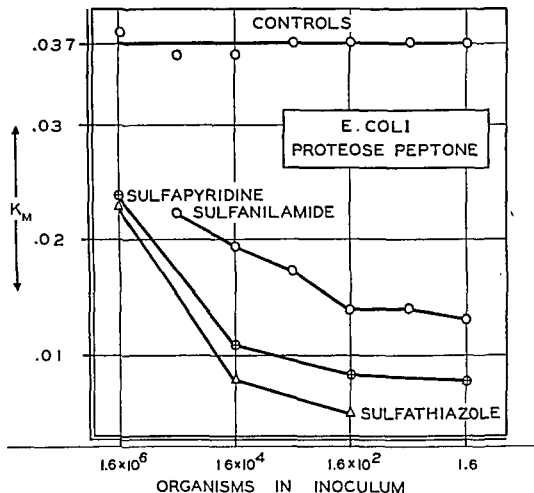


FIG. 1.  $K_m$  (VELOCITY CONSTANT OF GROWTH) AS A FUNCTION OF SIZE OF INOCULUM Medium PPFG. All drugs at  $10^{-3}$  molar

of  $K_m$ . Similar results have been reported for the streptococcus (17) and Pneumococcus Type III (18).

Theoretically, it is possible for variations in the size of inoculum to alter the action of the drug for other reasons. The organisms might destroy the drug, and thus a large inoculum could decrease its concentration. Using the method of Marshall (19), we have found no significant change in drug concentration during bacterial growth. Also, the effect of the drug would be diminished if the bacteria secreted an antagonist (e.g., *p*-aminobenzoic acid)



into the medium. As the size of the unwashed inoculum increased, the concentration of antagonist would approach and reach effective levels. On the other hand, size of inoculum cannot affect the action of non-diffusible

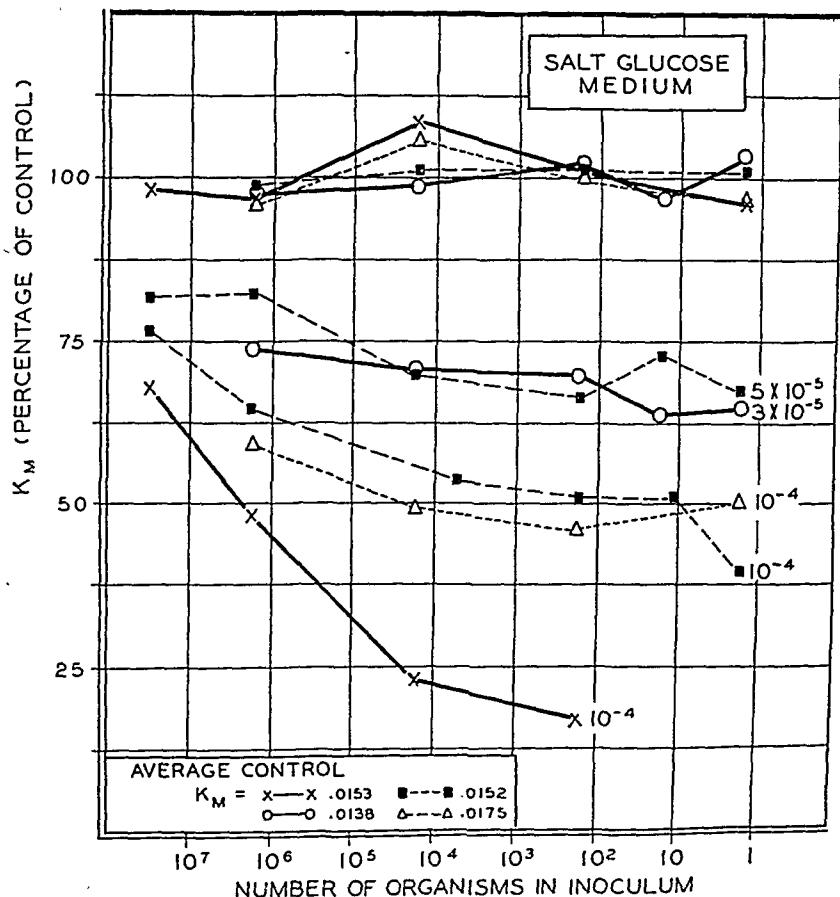


FIG. 2.  $K_M$  AS A FUNCTION OF SIZE OF INOCULUM

Medium SG. Four experiments are plotted in the figure. In each experiment  $K_M$  was determined for various sizes of inoculum in the absence (controls) and presence of sulfanilamide. The mean value for the controls in each case was determined, set equal to 100, and the other points plotted as percentages of this. Each experiment is characterized by a separate symbol.

antagonists, since their concentration per cell would not be increased by larger inocula. We have been unable to find any antagonist in the medium from cultures of *E. coli*, in agreement with the experience of MacLeod (11) and of Lowell, Strauss and Finland for *Pneumococcus Type III* (18). On the



other hand, positive results have been obtained following the growth of *Staphylococcus aureus* and *Pneumococcus Type I* (11) and *Brucella abortus* (2).

### Time

The role of time in the development of sulfanilamide inhibition is best illustrated by plotting  $K_s$  (as percentage of control) against time elapsed since the introduction of the drug. This has been done in figures 4 and 5 for the

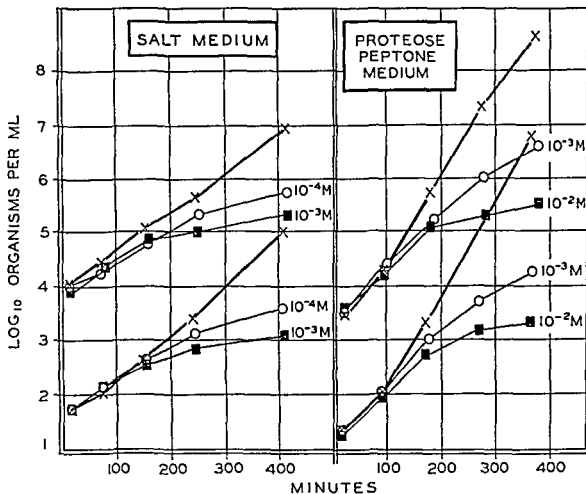


FIG. 3. THE COURSE OF SULFANILAMIDE INHIBITION DETERMINED BY VIABLE COUNTS  
hundred times  
Although the

data given in figure 3. These and other experiments show that usually between sixty and a hundred minutes must pass before a measurable inhibition occurs, both by viable count and photometry. After this latent period, the inhibition develops more or less rapidly during the next three or four hours, depending upon the medium and the drug concentration, and at the higher concentrations it continues to increase even after this.

The same results are obtained with very large inocula, though the experi-



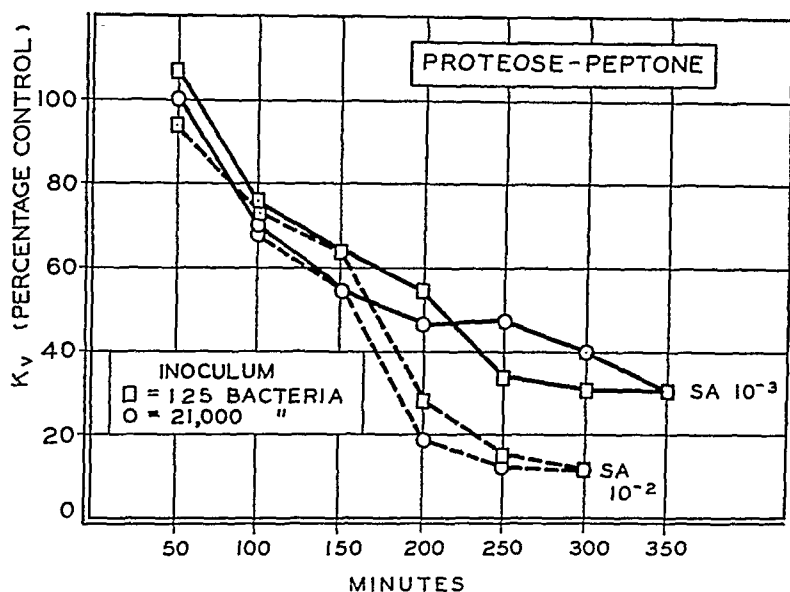


FIG. 4. THE COURSE OF SULFANILAMIDE INHIBITION IN A RICH MEDIUM (PPFG)  
 The velocity constant of growth  $K_v$  (as percentage of control) is plotted against time

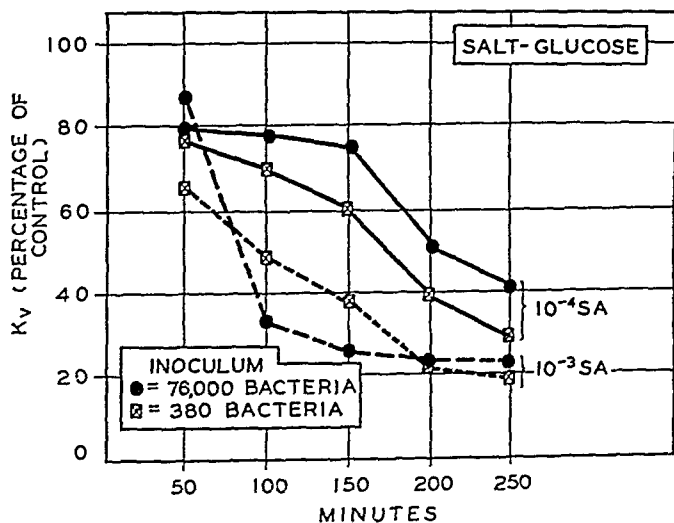


FIG. 5. THE COURSE OF SULFANILAMIDE INHIBITION IN A POOR MEDIUM (SG)  
 The velocity constant of growth  $K_v$  (as percentage of control) is plotted against time



ment is cut short because the medium becomes a limiting factor. Figure 6 shows the course of growth determined photometrically for an inoculum of  $1.7 \times 10^8$  organisms in SG. There was no inhibition during the first 85 minutes, after which it developed gradually during three hours.

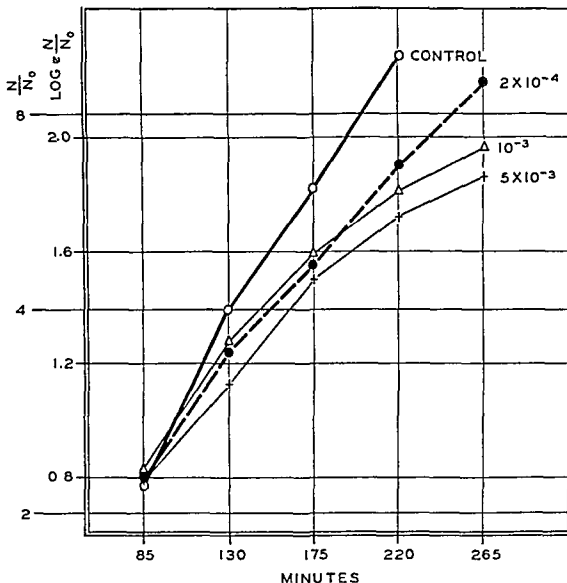


FIG. 6. COURSE OF SULFANILAMIDE ACTION FOLLOWED PHOTOMETRICALLY

The initial population density  $N_0$  was  $2.25 \times 10^7$  organisms per cc. The slope  $K$  is the velocity constant of multiplication. No inhibition is seen before 85 minutes, after which its development varies with the drug concentration.

The latent period has been observed, at least qualitatively, by most workers. One explanation, at first discarded, but recently championed by Libby (20) is that "the action of the sulfonamide drugs is a bactericidal one, and that this effect takes place immediately on a small percentage of susceptible organisms."

This hypothesis implies that the rate of multiplication is not gradually



slowed by the drug, but that the apparent slowing is due to the death of the more susceptible cells while the more resistant continue to divide at the normal rate. Hence, if lysis of the dead organisms does not take place immediately, there should be a smaller percentage of viable organisms in the inhibited culture than in the control. Experiment, however, does not support this contention. For example, the mean percentage inhibition of the growth rate for a culture in  $10^{-3}$  M sulfanilamide from the second to the third hour was found to be 41 by photometric count, 46 by viable count, 57 by direct count in the hemocytometer and 53 by oxygen consumption. In spite of this, the ratio of viable cells (determined by plating) to total cells (determined either by direct counting or photometrically) for the inhibited culture was within 10 per cent of that for the control. Theoretically, 28 per cent of the population should have been dead if the dead organisms remained visible for 35 minutes. This percentage should increase to 46 as the period between death and lysis is prolonged.

The latent period is readily explained by the *p*-aminobenzoic acid hypothesis when this is extended to include the exhaustion of reserves. Certain substances essential to growth and multiplication are probably stored in the bacterial cell. The sulfonamides either directly or indirectly inhibit the rate of their synthesis. Inhibition would not occur until the reserves had been depleted and the rate of synthesis had become a limiting factor. Inhibition would then gradually develop. The reasons for this choice of mechanism have been indicated in the introduction and will be more fully discussed later.

### *Concentration of sulfonamide*

The relation between  $K_m$  and drug concentration in SG is shown in figure 7, and in PPFG in figure 8. To make the results independent of the size of inoculum (see *Size of inoculum*), one of about 150 organisms was used. Each experiment was repeated and confirmed. Inspection of figure 7 shows that  $K_m$  in a rough way tends to be a semi-logarithmic function of the drug concentration in medium SG. In PPFG, however, the relation is more complicated, and the drugs are considerably less potent. The rough semi-logarithmic function holds until  $K_m$  has fallen to approximately 25 per cent of the control value. A plateau is then reached so that a twenty-five-fold increase in the concentration of sulfathiazole, sulfapyridine, or sulfadiazine depresses  $K_m$  by only 5 per cent. This plateau includes the range 2.5 to 25 mgm. per cent for sulfapyridine and sulfadiazine, and about 1 to 10 mgm. per cent for sulfathiazole; sulfanilamide, however, has only a slight tendency to show it. Following the plateau, the effect of the drugs is again proportional to the concentration.

Some of the peculiarities in the relationship between drug concentration and effect are shown by the data in table 1. The chief conclusions may be summarized as follows.



(1) The greatest differences in potency among the drugs are seen in SG, the least in PPFG when compared at concentrations necessary to produce large inhibitions.

(2) The relative activity of the drugs varies with the medium (note the difference between sulfathiazole and sulfadiazine in SG as compared to that in PPFG), and with the point selected for comparison (compare the columns in table 1 giving the concentrations necessary to produce a 30 or 85 per cent inhibition in PPFG). Thus, in SG a thirty-fold (or less) rise in drug concentration will increase the inhibition from 5 to 85 per cent, whereas in PPFG

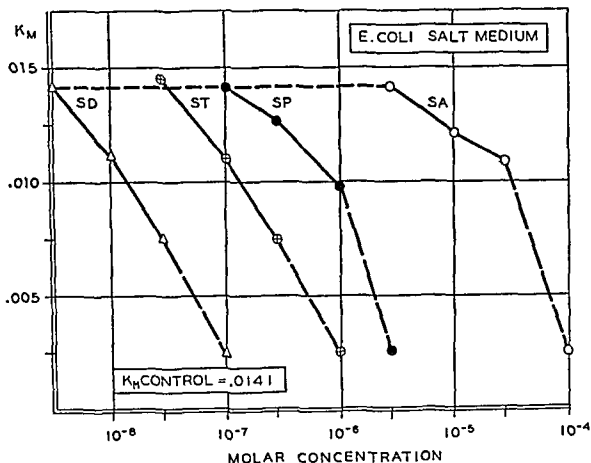


FIG. 7.  $K_M$  AS A FUNCTION OF DRUG CONCENTRATION IN MEDIUM SG

a hundred-fold rise is necessary for sulfanilamide, and a 500-1000 fold rise for sulfapyridine and sulfathiazole.

(3) The plateaus in the concentration curves occur in the therapeutic range of blood levels. This is of interest since it may explain the clinical experience that therapeutic effect (e.g., in the treatment of pneumonia with sulfapyridine or sulfathiazole) may be independent of the dose or blood concentration over a considerable range; it suggests that this would be the case in the treatment of *E. coli* infections.

To explain the foregoing, we suppose that (a) PPFG contains one or more



antagonists for the sulfonamides, (b) a particular drug directly or indirectly affects an increasing number of metabolic systems as its concentration is raised, and (c) some drugs affect more loci than others. Thus (a) explains the greater concentration required in PPFG and also the appearance of plateaus in the curves for sulfapyridine, sulfathiazole and sulfadiazine, (b) explains the increase in inhibition when the concentration is raised high

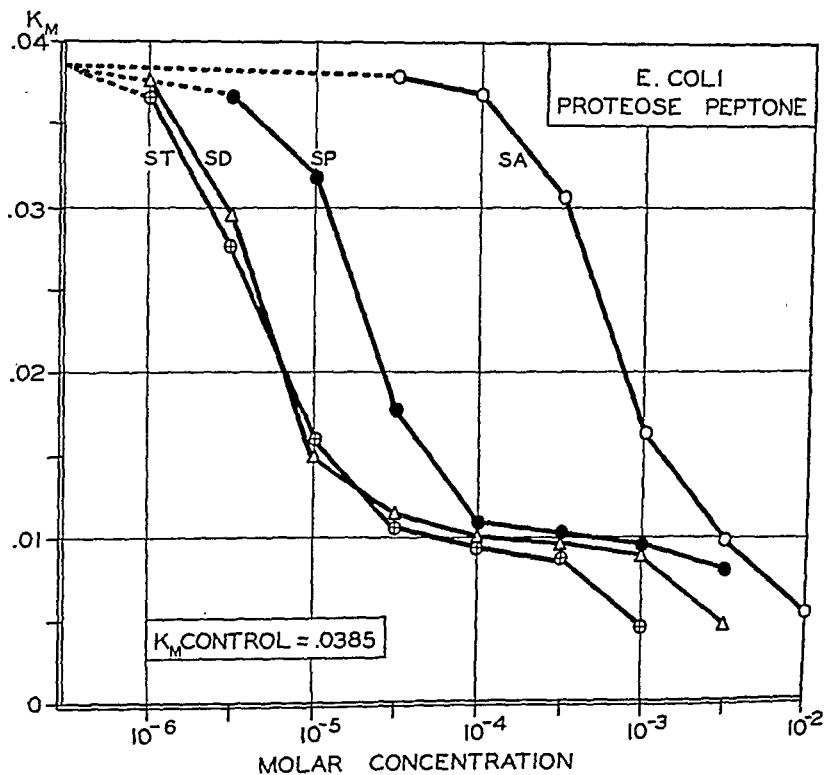


FIG. 8.  $K_M$  AS A FUNCTION OF DRUG CONCENTRATION IN MEDIUM PPFG

enough to escape the plateau, and (c) explains why the sulfanilamide curve fails to show a plateau.

#### *Culture medium*

Since most of the data pertaining to the effect of the culture medium have already been presented, they will be but briefly collected and summarized here. The control growth is roughly two and one-half times more rapid in PPFG than in SG. The concentration of drug necessary to inhibit  $K$  by 50 per cent is about twenty times greater for the richer medium. Figures 4 and



5 show that the course of the inhibition with respect to time is very similar in the two media, although the bacteria are dividing at widely different rates. The richer medium contains antagonists (see *Concentration of sulfanilamide*) which seem to be more effective against sulfanilamide than the other drugs.

### Respiration

The respiration of bacteria during the resting and growing phases was studied by the Warburg technique. Washed cells were suspended in a buffer solution (pH 7.4) composed of 4 grams of NaCl, 0.2 grams of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.344 grams of  $\text{Na}_2\text{HPO}_4$ , 0.0785 grams of  $\text{KH}_2\text{PO}_4$  and water to make 100 cc. solution. Glucose or lactate was added to give a final concentration of 200 mgm. per cent. The oxygen consumptions of such suspensions were not changed by the addition of sulfonamide ( $10^{-2}$  M and  $10^{-4}$  M sulfanilamide or  $10^{-3}$  M and  $10^{-6}$  M sulfathiazole) even after a three hour period of contact

TABLE 1

*Relative concentrations of sulfonamides required to depress  $K_m$*

In each case the concentration of sulfanilamide is set equal to 1; its absolute value is given in brackets. The culture medium is indicated.

DRUG	REDUCTION IN $K_m$		
	30 per cent		85 per cent
	SG	PPFG	PPFG
Sulfanilamide	1 ( $3 \times 10^{-4}$ M)	1 ( $5 \times 10^{-4}$ M)	1 ( $5 \times 10^{-4}$ M)
Sulfapyridine.	$\frac{1}{30}$	$\frac{1}{25}$	$\frac{1}{2}$
Sulfathiazole	$\frac{1}{300}$	$\frac{1}{150}$	$\frac{1}{10}$
Sulfadiazine	$\frac{1}{3000}$	$\frac{1}{150}$	$\frac{1}{5}$

with the drug. The R.Q. determined with the glucose substrate was also unchanged.

The oxygen uptake of growing cells was measured in SG medium. The absorption of carbon dioxide in the Warburg vessels did not interfere with growth. The rate of growth was measured by the usual logarithmic formula using (instead of number of organisms) the rate of oxygen uptake as calculated from the slope of the oxygen uptake of the vessels. Cells were counted by the plating technique. Table 2 gives the results of several experiments in which the organisms were inhibited by various concentrations of the sulfonamides. At the lower sulfonamide concentrations, the oxygen uptake per bacterium does not fall appreciably even though the growth constant  $K$  is diminishing. Similar findings have been reported for *E. coli* (21) and for *B. abortus* (22). Later in both concentrations, there is a fall in the rate of oxygen consumption per cell but this lags behind the fall in the growth constant. The R.Q. was unchanged in the presence of  $10^{-3}$  M and  $10^{-4}$  M sulfanilamide.



It is difficult to interpret these results because of the number of variables present: number of dead organisms, number of organisms which can utilize oxygen but cannot divide, the effect of various growth rates on the extra oxygen consumption per cell incident to growth, the size of the cells and the changes in the medium caused by the organisms. Some of the variables relating to the proportion of viable cells to total cell mass may be eliminated by the use of photometric measurement of the bacteria. As shown in table

TABLE 2

*The effect of the sulfonamides on the growth constant and respiration per cell of E. coli growing in SG medium*

CONCENTRATION OF DRUG	TIME AFTER INOCULATING	K	OXYGEN UPTAKE PER HOUR PER 10 <sup>7</sup> CELLS	
			From plate count	From photometric count
	<i>minutes</i>		<i>mm.</i>	<i>mm.</i>
0	100	0.021	2.7	2.1
10 <sup>-4</sup> M SA	100	0.014	2.6	1.9
10 <sup>-3</sup> M SA	100	0.010	1.7	1.3
0	150	0.019	2.2	1.7
10 <sup>-4</sup> M SA	150	0.008	1.9	1.4
10 <sup>-3</sup> M SA	150	0.00	1.4	1.0
0	120	0.018	2.3	1.9
3 × 10 <sup>-6</sup> M SP	120	0.016	2.2	1.4
3 × 10 <sup>-5</sup> M SP	120	0.009	1.0	
0	160	0.016	1.9	1.5
3 × 10 <sup>-6</sup> M SP	160	0.015	1.8	1.3
3 × 10 <sup>-5</sup> M SP	160	0.006	1.1	0.8
0	110	0.017	2.5	1.4
10 <sup>-6</sup> M ST	110	0.014	2.4	1.3
10 <sup>-5</sup> M ST	110	0.008	1.7	1.2
0	160	0.017	1.8	1.6
10 <sup>-6</sup> M ST	160	0.013	1.9	1.4
10 <sup>-5</sup> M ST	160	0.004	1.7	1.1

2, the oxygen uptake per photometric cell varies in a similar fashion to the uptake per viable cell.

In spite of the limitations on interpretation, it appears from the experiments on the "resting" and dividing cells, that the sulfonamides do not have a direct influence on the action of the respiratory enzymes. As growth is slowed, the synthesis of respiratory enzymes is likewise disturbed so that, for a while at least, the content of respiratory enzymes in the bacterium remains unchanged. Later it may be that the synthesis of the enzymes is more affected than the synthesis of cell mass and the rate of division. Nevertheless, it is not pri-



marily for want of available energy that growth slows but for the want of its utilization in the mechanisms of the bacterium for synthesis and division.

TABLE 3

Organisms were placed into media with and without sulfonamide and maintained for three hours at 5°C. Drugs were then added to the tubes lacking them and the cultures were incubated at 37°C. The time in minutes required to reach a certain optical density is recorded. To reach this endpoint, roughly four divisions were required in SG and nine in PPFG.

MEDIUM	DRUGS ADDED BE- FORE OR AFTER ING	CONCENTRATION OF DRUG									NO DRUG
		Sulfanilamide			Sulfapyridine			Sulfathiazole			
		M 10 <sup>-3</sup>	M 10 <sup>-4</sup>	M 10 <sup>-5</sup>	M 3×10 <sup>-5</sup>	M 3×10 <sup>-6</sup>	M 3×10 <sup>-7</sup>	M 10 <sup>-4</sup>	M 10 <sup>-5</sup>	M 10 <sup>-6</sup>	
SG	Before		360	230		340	240		380	275	205
SG	After		320	230		325	240		365	270	205
PPFG	Before	355	230		275	190		310	240		185
PPFG	After	355	225		320	190		275	230		175

TABLE 4

Organisms were placed in a saline phosphate buffer and additions of drug, glucose or ammonium chloride were made before or after a three hour incubation at 37°C. as indicated. Following the second set of additions, the time required to reach a certain optical density was measured and is recorded in minutes. To achieve this density, roughly 4.5 divisions were necessary in the cultures where glucose was added after the preliminary incubation and roughly 3.5 divisions in the remaining set.

ADDITIONS BEFORE PRELIMINARY INCUBATION			ADDITIONS AFTER PRELIMINARY INCUBATION			TIME TO REACH ENDPOINT						Control without drug
Drug	Glu- cose*	NH <sub>4</sub> Cl*	Drug	Glu- cose	NH <sub>4</sub> Cl	SA 10 <sup>-3</sup>	SA 10 <sup>-4</sup>	SP 3 × 10 <sup>-5</sup>	SP 3 × 10 <sup>-6</sup>	ST 10 <sup>-4</sup>	ST 10 <sup>-5</sup>	
+	0	+	0	+	0	540	310	555	345	690	405	280
0	0	+	+	+	0	535	310	580	350	705	420	
+	0	0	0	+	+	610	320	620	355	750	415	
0	0	0	+	+	+	570	320	590	360	810	420	285
+	+	0	0	0	+	380	265	420	270	465	315	
0	+	0	+	0	+	330	280	390	265	435	300	235

\* Final concentration of glucose and of ammonium chloride was 200 mgm. per cent.

#### Metabolic state

Although the characteristic inhibition of the sulfonamides can only be measured during growth, the question arises as to whether drug can interact



with the resting cell. An analogy might be drawn with the photographic film, which must be developed in order to show the effect of previous exposure to light. Likewise the resting cell may interact with the drug, but the effect could not be seen until growth had occurred. To test this possibility bacterial suspensions, whose growth was prevented by the withdrawal from the medium of glucose or ammonia, or by low temperature, were exposed to the sulfonamides for periods of one to three hours. Sulfonamide was then added to those cultures lacking it and growth was initiated by the addition of the missing factor. Some protocols are summarized in tables 3 and 4. It will be observed that the presence or absence of sulfonamide during the preliminary incubation period did not affect the development of inhibition during the subsequent period of growth.

The interaction between the sulfonamide and the bacterium, therefore, depends upon some reaction associated intimately with growth. Directly or indirectly, this reaction in *E. coli* is initiated by ammonia, glucose, salts, and a sufficiently high temperature.

#### DISCUSSION

The type of theory used to describe the various data depends largely on the mechanism selected to explain two principal facts; why time must elapse before the action of the drugs is apparent, and why certain processes intimately associated with growth must occur during this period. The possibilities which suggest themselves are:

- I. Reactions involving sulfonamide directly.
  - A. Penetration of the drug to the sensitive locus.
  - B. Reaction of the drug at the sensitive locus.
  - C. Formation of an active agent from the drug.
- II. Reactions involving sulfonamide indirectly.
  - A. The lack of essential substances whose synthesis by the cell is inhibited by sulfonamide.

To choose among these various possibilities is difficult. Temperature and other special conditions often affect the penetration of drug to the sensitive locus. Nothing is known about the speed of reaction, or the type of reaction, which occurs at the sensitive locus, and therefore we cannot eliminate these as possibilities. Although the formation of an active agent has not been proved, the possibility remains that one is formed and remains within the bacterium (23, 24).

The choice of a mechanism, then, becomes partly a matter of prejudice. Because we feel the need of a working hypothesis, and because we believe an extension of the *p*-aminobenzoic acid theory is the most useful point of view experimentally, we have adopted the fourth possibility listed, which was briefly mentioned under *Time* as the exhaustion of reserves. This assumes that certain substances essential to growth and multiplication are stored in



the cell, and that the rate of their synthesis is inhibited by the sulfonamides. The action of sulfonamides cannot be seen, therefore, until these reserves are depleted and the rate of synthesis has become a limiting factor. This accounts for the induction period, and also permits an inhibition varying from zero to 100 per cent. Since the reserves are used up only in the process of growth, for example in the synthesis of protein, the necessity for growth during the induction period is obvious.

Provisionally, we picture the whole process in *E. coli* as follows: The addition of nitrogen in a useful form to cells suspended in salt and glucose increases the respiration per cell. This extra energy production is not inhibited by the sulfonamides; its purpose is to run the syntheses necessary for growth and multiplication. Among the syntheses there is a special group *X* which requires *p*-aminobenzoic acid as a catalyst. The sulfonamides compete with *p*-aminobenzoic acid in these reactions, thereby inhibiting the synthesis of *X*. Their effectiveness depends upon the bacterial species, and even upon the strain, for reasons not understood and lumped under the term *resistance*. The action of the drugs is not immediately apparent because the cells contain stores of *X*. The degree of inhibition finally reached depends upon the extent to which the synthesis of *X* is inhibited, and upon the amounts of *X* and *p*-aminobenzoic acid in the culture medium. *X* represents a class of substances *a*, *b*, *c*, *d*, *e*. At low concentrations of the drug, the synthesis of *a*, *b*, and *c*, may be stopped, but not of *d* and *e*. Proteose-peptone medium which contains *a*, *b* and *c* can therefore antagonize low concentrations of the drug; but it cannot antagonize high concentrations which also prevent the synthesis of *d* and *e*.

We would expect the foregoing to be generally true for all sulfonamide-sensitive bacteria, subject to three limitations.

(1) Many bacteria cannot be grown in simple media of known composition. Results obtained with them are always subject to the complication that antagonists may be present which necessitate working with higher drug concentrations than otherwise.

(2) Evolution or specialization has frequently resulted in a loss of synthetic ability or has changed internal metabolic relationships. Thus, for example, sulfapyridine may inhibit the synthesis of cozymase in species *A*, but this could never occur in *Hemophilus parainfluenzae* which requires cozymase as an essential growth factor.

(3) Some of the sulfonamides may inhibit reactions which others cannot, and which *p*-aminobenzoic acid does not antagonize, as for example, the action of sulfapyridine on the respiration of the dysentery bacillus (14). The effect of these additional inhibitions would have to be superimposed upon the foregoing.

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possible, of course, that antagonists may function by increasing the production of p-amino benzoic acid or by opening new metabolic paths which eliminate the necessity for the reactions involving p-amino benzoic acid.

#### SUMMARY

1. Quantitative data on the growth and respiration of *E. coli* in different media, in the presence and absence of the sulfonamides, has been obtained. Sulfanilamide, sulfapyridine, sulfathiazole and sulfadiazine were employed.

2. The size of inoculum has no real effect on the results. The apparent effect is due to the limitations of the medium and the time course of the inhibition.

3. There is a latent period of sixty to ninety minutes before the action of the sulfonamides on the rate of growth is measurable. Following this, the inhibition develops gradually during the course of three or more hours, depending on the concentration of the drug and the medium.

4. The relationship between rate of growth and drug concentration is plotted. It is simple in a salt-glucose medium, but rather complex in one containing proteose-peptone. These complicated relationships make difficult the evaluation of drug potency and may partly explain the clinical experience that therapeutic result may seem to be independent of sulfonamide blood concentration.

5. They indicate that, as the concentration of the drug is raised, the number of reactions inhibited is increased, and that sulfanilamide cannot inhibit some reaction which is sensitive to the other drugs.

6. At the bacteriostatically active concentrations studied, the sulfonamides do not primarily inhibit the respiration (per cell) of the resting or growing organism.

7. Incubation of non-growing cells with sulfonamide does not affect the subsequent development of inhibition when growth is initiated. Growth was prevented by low temperature, or by withholding ammonia or glucose.

8. A provisional theoretical description of the mode of action of the drug is given.

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# A METHOD FOR THE RAPID ISOLATION AND SPECTROGRAPHIC MEASUREMENT OF COCAINE FROM BRAIN TISSUE

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*From the Spectrographic Laboratory of the Medical Center and the Department of Surgery of the University of California Medical School*

Received for publication May 19, 1941

To extend that portion of an investigation in which experimental epilepsy (1) was induced by cocaine hydrochloride and the cocaine was detected in the cerebrospinal fluid by spectrochemical methods (2), it was essential to secure quantitative analyses, with less than 10 per cent error, sensitive to one milligram or less of cocaine isolated from approximately 10 grams of brain tissue. Most methods of analysis for cocaine have not dealt with restrictions so rigorous as the foregoing, since the analyst has been concerned with larger portions of tissue (100-500 grams) and the analyses have been qualitative or semi-quantitative for amounts of alkaloid less than 100 milligrams. The basic procedure of Stas (3) as modified by Otto (4) and Dragendorff (5) has remained the classical method for the isolation of organic poisons. In many instances, however, the large number and the nature of the operations required by this method render it unreliable for the quantitative isolation of compounds present only in trace amounts. This is particularly true in the case of the more easily hydrolyzed alkaloids, such as cocaine. In addition, the difficulty of accurate measurement of the isolated alkaloid increases disproportionately for minute amounts. Drastic methods of removing impurities may jeopardize the results. On the other hand, insufficient purification is probably responsible for the spuriously high results often quoted.

The evaluation of a method of isolation depends upon the reliability of the method of measurement of the isolated product. Indeed, it may be surmised that the differences of opinion (6, 7, 8) relative to the merits of different methods of isolation remain unresolved because of inadequacy of the methods of measurement employed. With the use of the ultraviolet spectrographic method, inconsistencies in the more recent methods of alkaloid isolation as advocated in the literature became evident. The process of isolation, therefore, was studied in detail and modifications were made which have not only resulted in an improved method of alkaloidal assay, but in addition may be applicable to the general problem of analysis of tissues.

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For purposes of study the method of assay of an alkaloid was considered under the following headings:

**PREPARATION OF THE TISSUE.** To obtain fine comminution, the tissue was cut into small pieces, immersed in liquid air and ground through a fine-bladed mincer with pieces of solid carbon dioxide. Loss of tissue was avoided by "washing" through with additional solid carbon dioxide. As the carbon dioxide sublimed off and the temperature of the tissue increased, the cells ruptured and complete maceration was attained. This technique not only effects a more nearly complete maceration than is attained by the usual mechanical methods, but also has the advantage of abruptly reducing the tissue metabolism and decomposition to a minimum at precise times.

**INITIAL ACID EXTRACTION OF THE ALKALOID AND REMOVAL OF THE EXTRACTIVE AGENT FROM THE RESIDUUM.** Acidified, absolute and even dilute alcohol have been found unsatisfactory for the initial extraction process when the amount of alkaloid is small (9). We accordingly confined ourselves to weakly acidified water. Impurities which were not removed as a result of omission of alcohol did not prove to be a source of error in the spectrographic assay of the alkaloid.

TABLE 1

*Analysis of cocaine from body fluids*

Trichloroacetic acid used as agent of precipitation and extraction

EXPERIMENT	AMOUNT OF COCAINE-HCl USED	AMOUNT OF BLOOD SERUM	YIELD
	mgm. per 24 cc. H <sub>2</sub> O		per cent
1	1	1 cc.	90
2	1	8 drops	96
3	1	3 drops	91
4	1	9 drops	90
5	1	37 drops	95
6	0*	1 cc.	5

\* Control.

Trichloroacetic acid has been advocated by Florence (7) and Stewart et al. (8) to simplify and expedite the initial extraction. Joanid (10) has found it less reliable than the Stas-Otto method and Daubney and Nickolls (11) have criticized its use. A spectrochemical evaluation of this method has shown it to be satisfactory for the analysis of body fluids (table 1), but inadequate in the case of tissues (table 2). It was found, however (table 3), that hydrolysis alone did not explain this loss.

The use of weak acetic acid as an extractive agent in conjunction with a protein precipitant, ammonium sulfate (11), was verified as applied to viscera (table 5), but required modification for the analysis of brain and other tissues. The necessity for modification lay in a double effect of the ammonium sulfate upon the procedure of extraction of the alkaloid from the macerate: 1. In the presence of an excess of ammonium sulfate, cocaine was not extracted by the acid filtrate<sup>1</sup> (table 4). 2. As the concentration of ammonium sulfate was decreased by addition of acid sufficiently to permit extraction of the alkaloid, the relative densities of the residual mass and extractive acid became such as to make them inseparable.

Starting with a slight excess of ammonium sulfate in the first extraction, we added

<sup>1</sup> This is true in spite of the fact that cocaine hydrochloride is readily soluble in a saturated ammonium sulfate solution.



amounts of acid which reversed the relationship between liquid and coagulum and permitted their easy separation upon centrifuging the second extraction. The procedure adopted was satisfactory for brain and other tissues. It has been used with equally good results on young mice including the hair and bones.

**PURIFICATION OF THE INITIAL EXTRACT BY CYCLIC CHLOROFORM-ACID EXTRACTIONS.**

*Extraction from aqueous solution with immiscible solvent.* This procedure removes excess fats and resinous matter. The acid of the previous stage was rendered alkaline

TABLE 2

*Results obtained with trichloroacetic acid as extracting agent*

AMOUNT OF COCAINE	KIND OF TISSUE	AMOUNT OF TISSUE	MEASURED YIELD	REMARKS
mgm.		grams	per cent	
1	Brain	25	40	Cold trichloroacetic used alone, 5 extractions. No emulsions
1	Brain	30	33	Trichloroacetic acid used at 65°C. Slight emulsion broken down by centrifuging
1	Liver	27	33	Same as experiment above. No emulsions. Expeditious procedure
10	Liver	27	31	Same as above except ten-fold increase in alkaloid. No emulsions

TABLE 3

*Hydrolysis of cocaine by trichloroacetic acid*

TEMPERATURE	TIME	COCAINE RECOVERED	ESTIMATED ERROR
°C.	min.	per cent	per cent
60	45	74	10
20	60	102	10

TABLE 4

*Results obtained using saturated ammonium sulfate solution alone as extracting agent.  
1 mgm. of cocaine hydrochloride used*

KIND OF TISSUE	GRAMS OF TISSUE	YIELD	NUMBER OF EXTRACTIONS
Brain.....	27	Negligible	1
Brain....	27	Negligible	2
Liver ....	30	Approx. 5%	4

with concentrated ammonium hydroxide in preference to sodium hydroxide because cocaine is almost insoluble in an excess of ammonia, whereas precipitation by other agents caused traces of decomposition to appear (12). Because cocaine readily decomposes in alkaline solution, its extraction should be carried out at a pH less than 9 (13). Chloroform was preferred to ether because it is less soluble in water.

The efficacy of this procedure and the optimum time of agitation were determined by measuring the yields spectrographically following single extractions of varying durations. The upper curve of figure 1 demonstrates that one agitation of five minutes'



duration sufficed to extract most of the cocaine. Three such extractions, therefore, were considered adequate for all experimental conditions.

The lower curve of figure 1, made from experiments on the same test solution after standing one week, demonstrates the decomposition of the cocaine in normal acid solution. This degree of loss agrees with other observations (13). The importance of neutralizing as quickly as possible intermediate solutions of cocaine is evident.

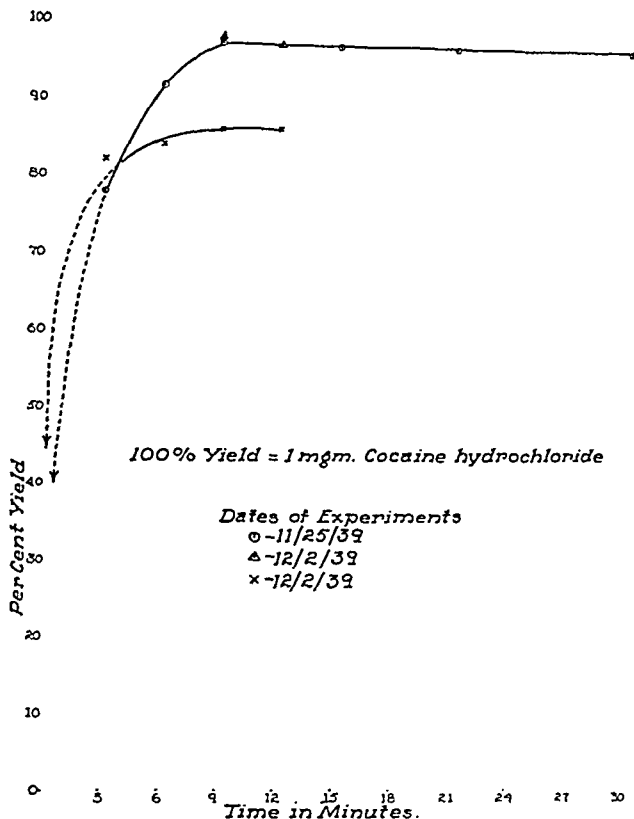


FIG. 1. YIELD OF COCAINE WITH RESPECT TO TIME OF EXTRACTION

The upper curve indicates the yield obtained with immediate extraction. The lower yield (lower curve), obtained with identical solutions and the same procedure 1 week later, was shown to result from the decomposition of cocaine in acid solutions and emphasizes the importance of neutralizing such solutions as quickly as possible in the process of extraction.

*Extraction from immiscible solvent with acid-emulsions.* In this stage the aqueous solution must be made sufficiently acid to convert all of the free alkaloid into the water-soluble salt. Although emulsions formed in the preceding stage they were particularly troublesome in the acid extracts of the chloroform filtrate. Incomplete separation of phases due to the formation of emulsions have long been recognized as an important source of error in the quantitative isolation of alkaloids (14). Centrifugation of all



separable phases proved valuable in overcoming this serious difficulty. The tendency to form emulsions was greater with brain tissue than other tissues and was more pronounced for those procedures of extractions that gave the highest yields.

*Extraction from aqueous solution with immiscible solvent.* Since the aqueous phase was invariably clear and produced no emulsions upon agitation with chloroform this step was the final stage necessary in the cyclic process of extraction. When solutions containing known amounts of cocaine are subjected to three successive extractions carried out in the manner described, we obtained a 95 per cent yield with an estimated error of 5 per cent.

*EVAPORATION OF THE IMMISCIBLE SOLVENT.* Because chloroform is not a satisfactory spectrographic solvent, it was necessary to evaporate it and redissolve the cocaine in dilute acid (N/25 HCl). The process of evaporation must not be accompanied by overheating of the alkaloid. Evaporation aided by a fan in an open container partially immersed in a water bath proved to be more rapid than various other techniques which were tried. Twenty-five cc. of chloroform were evaporated to dryness safely in 10 minutes. Because chloroform tends to form unstable compounds with many alkaloids the evaporated residue was treated with a little ether, which in turn was evaporated.

**SUMMARY OF PROCEDURE ADOPTED FOR EXTRACTION OF COCAINE FROM BRAIN TISSUE.** Twelve grams of brain were frozen with liquid air and minced with carbon dioxide, as described above under "Preparation of the tissue." Five cubic centimeters of distilled water, 1.5 cc. of glacial acetic acid at about 50°C. and approximately 0.66 gram of ammonium sulfate per gram of brain were added to the macerate, thoroughly mixed with the aid of a thermometer and slowly heated to 67°C.

*Initial acid extraction.* The mixture was centrifuged at approximately 2,000 r.p.m. for 5 minutes in a thick-walled tube. The clear subnatant fluid was removed through a hole made in the tightly packed supernatant mass by means of the thermometer and filtered in a Buchner funnel without suction. The filtrate was transferred to a separatory funnel and neutralized (phenol red indicator) with concentrated ammonium hydroxide. The container originally holding the tissue was washed with 20 cc. of warm 1 per cent acetic acid, which was added to the residue in the centrifuge tube. Thorough mixture and heating to 65 degrees C was repeated for a period of 5 minutes. Ten minutes of high speed centrifugation produced a complete separation of the turbid supernatant fluid and subnatant residue. The supernatant fluid was filtered as before and the new filtrate was added to the filtrate in the separatory flask. Poor filtration resulted when any residue was left on the filter paper and was avoided by its removal with a policeman rod. Three further extractions of warm 1 per cent acetic acid in amounts of 13 cc., 12 cc., and 10 cc., respectively, were performed in the same manner, except that shorter periods of centrifugation (5 minutes) were required. Successive extractions became less turbid and the final one was clear.

*First stage of purification.* The filtrate (approximately 70 cc.) was made alkaline with ammonium hydroxide. A white, flocculent precipitate appeared and the turbidity disappeared. Twenty-five cc. of chloroform were added to



the filtrate and thoroughly mixed with it by shaking for 5 minutes. A slight emulsion appeared. The supernatant chloroform and intermediate emulsion were drawn off into a tube and centrifuged at high speed for 5 minutes. A clear separation resulted. A disc of white precipitate separated the supernatant aqueous solution from the chloroform in the bottom of the tube. The chloroform was removed by pipette and filtered into a secondary separatory flask. Two additional chloroform extractions were made in the same way on the alkalinized, aqueous solution.

*Second stage of purification.* The combined chloroform filtrates were extracted four times with 20 cc., 15 cc., 10 cc. and 10 cc. of 1-normal sulphuric acid. Emulsions, which particularly tended to form at this stage, were minimized by the addition of ethyl alcohol in the volumetric ratio of one part of alcohol to six parts of acid. High speed centrifugation for 5 minutes further broke down the emulsions and gave good separations. The supernatant acid layers, now quite clear, were filtered in turn into another separatory flask and immediately neutralized with concentrated ammonium hydroxide.

*Final stage of purification.* The combined filtrates were rendered alkaline and again extracted three times with 25, 20 and 20 cc. of chloroform in the same manner as described for the first stage of purification. No emulsions appeared in this stage. The chloroform of each extraction was filtered into a beaker and evaporated almost to dryness over a hot bath and under a fan before the chloroform of the next extraction was added. The residue was dissolved in 2 cc. of ether, and this in turn was evaporated to dryness.

THE MEASUREMENT OF THE ISOLATED ALKALOID. The principle of the method of measurement is based on the properties of the optical absorption of the alkaloid, i.e., the absorption coefficient or extinction coefficient (15), which varies over a series of wave lengths in a characteristic fashion.

Spectrochemically cocaine hydrochloride is measured more readily than basic cocaine. This is due, in part, to the fact that solvents, such as chloroform, unless specially purified, have absorption in the same region of the spectrum as cocaine. After the chloroform and ether of the last stage of purification have been removed, the solution to be measured was made by adding 5 or 10 cc. of N/25 hydrochloric acid.

Two absorption bands, one centering at  $\lambda 2750$  and a somewhat stronger one centering at  $\lambda 2330$ , are characteristic of cocaine hydrochloride (fig. 2). A parallel beam of ultraviolet continuum was obtained from a high voltage hydrogen discharge tube,<sup>2</sup> cooled by water and controlled for constant current output. The beam was passed through the test solution in a Baly tube or cylindric quartz absorption cell, 4 cc. in volume and 2 cm. long, and focused on the slit of a small quartz spectrograph of 17 angstrom units per millimeter at  $\lambda 2700$  (Hilger E 484) or a medium quartz spectrograph of 7 angstrom units

<sup>2</sup> The low voltage D.C. glow source made by Bartol Foundation has also been used. Although it gives a very satisfactory continuum, it was found to be less constant.



per millimeter at  $\lambda 2700$  (Hilger E 498). Eastman plates (#33) were used for solutions whose concentrations were between 0.02 gram and 0.004 gram per cent, and plates whose emulsions were particularly sensitized to the ultraviolet rays (Eastman No. I O u.v.) were used for concentrations between 0.003 and 0.001 gram per cent.

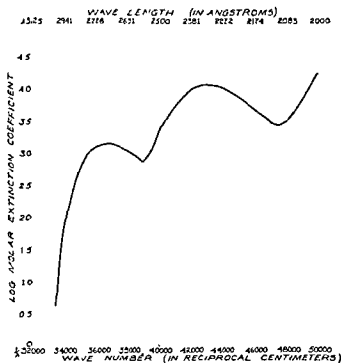


FIG. 2. ABSORPTION SPECTRUM OF COCAINE HYDROCHLORIDE IN WATER (AFTER INTERNATIONAL CRITICAL TABLES)

The quantity of light absorbed at a given wave length is a function of the concentration of the solution. According to the laws of Beer and Lambert, valid for dilute solutions, this relationship may be written as follows:

$$C = 1/\epsilon d \log_{10} \frac{I_0}{I}$$

in which

$C$  is the concentration of the solution (moles per liter),

$\epsilon$  is the molecular extinction coefficient,

$d$  is the length of absorbing solution through which the light passes,

$I_0$  is the intensity of light incident upon the solution, and

$I$  is the intensity of light after passing through the solution.

$\log_{10} I_0/I$  is defined as the optical density,  $D$ .

Thus, with a cell of fixed length ( $d$ ), since  $\epsilon$  is a determinable constant for the particular molecular structure, the concentration is a direct function of the optical density,  $D$ .

The method of screens devised by Harrison (16) was employed to establish the relationship between the optical densities of the solutions and the corre-



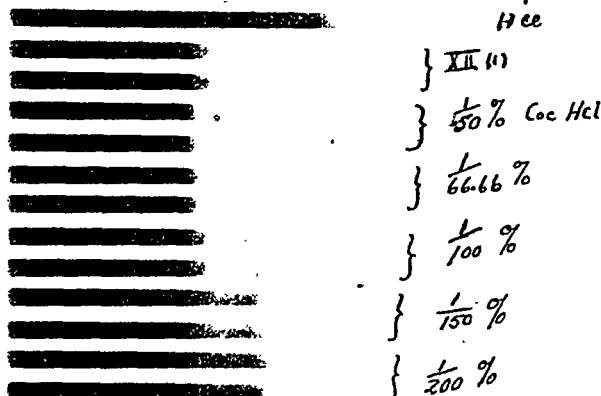


FIG. 3a. DUPLICATE SPECTROGRAMS OF UNKNOWN SOLUTIONS XII (1) AND OF SELECTED SERIES OF KNOWN CONCENTRATIONS OF COCAINE HYDROCHLORIDE

The spectrogram of the solvent (N/25 HCl) is shown singly. Note the change in the transparency with decreasing concentrations of cocaine hydrochloride, which are measured 50 angstroms to the right of the fiducial line at 2830 angstroms.

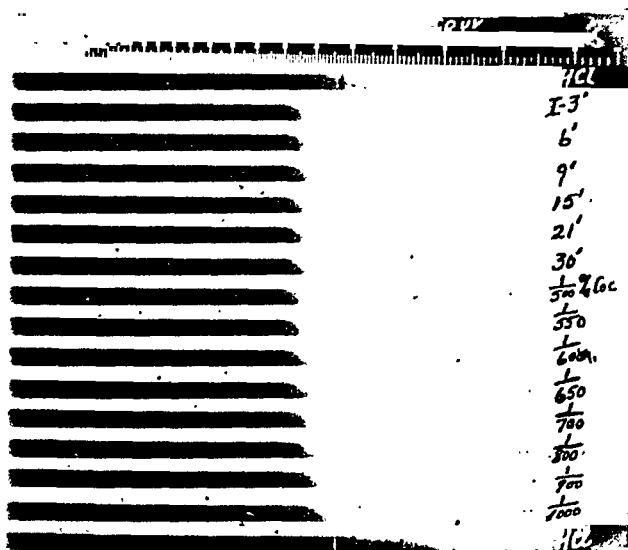


FIG. 3b. SPECTROGRAMS ON 10 U.V. EMULSIONS ILLUSTRATING THE USE OF THE STRONGER ADSORPTION BAND OF COCAINE HYDROCHLORIDE AT  $\lambda$  2250 FOR MEASURING CONCENTRATIONS OF COCAINE BETWEEN 0.003 AND 0.001 GRAMS PER CENT



sponding transparencies on the photographic plate in the region of absorption. We have found it more convenient, however, to calibrate the plate transparencies (directly) with respect to known concentrations of cocaine. Duplicate exposures of each unknown solution were made successively and followed

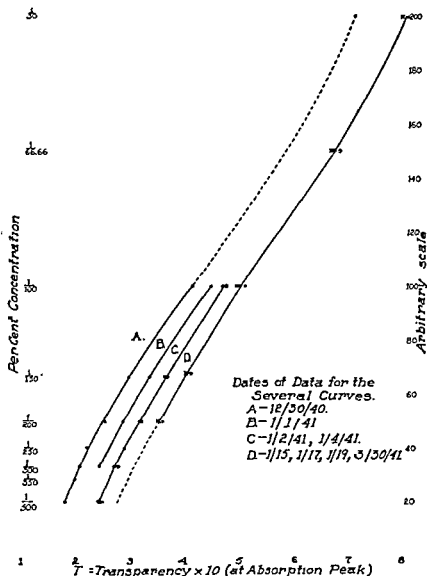


FIG. 4. TYPICAL CALIBRATION CURVES

The concentration of the standard solutions (ordinate) is plotted against plate transparency (abscissa) at the wave length of maximum absorption. The concentration of the unknown solutions is determined by such calibration of the concentration-transparency relationship of the microphotograms deviating

by a selected series of duplicate exposures of solutions of known concentrations (fig. 3). The transparency at the wave length of maximum<sup>3</sup> absorption was measured by the Zeiss recording microphotometer in the Department of

<sup>3</sup> It follows from Beer's law that D varies most rapidly with the changes of concentration at the wave length of the peak of absorption.



Physics of the University of California. The values of transparency, determined in this manner, were then plotted against the concentrations of the known solutions. A calibration curve thus was obtained from which the concentrations of the unknown on the same plate were interpolated graphically. Figure 4 shows several calibration curves used in the determination of cocaine in the experiments described in this paper.<sup>4</sup> It is essential that each plate be calibrated in this way. Duplicate spectrograms were found to deviate 3 per cent from the mean when measured by this method.

With careful attention to the details of the procedure, consistent, high yields were achieved with this method (table 5).

Yields considerably greater than 100 per cent have been reported and probably represent a failure in the method of measurement to distinguish impuri-

TABLE 5  
*Analysis of tissue using 1 per cent acetic acid and ammonium sulfate*

AMOUNT OF COCAINE	KIND OF TISSUE	AMOUNT OF TISSUE	YIELD	REMARKS
mgm.		grams	per cent	
1	Liver	25	72	Duplication of procedure described by Daubney and Nickolls (11). Emulsions not completely broken down as with improved procedure
1	Brain	25	85	Procedure intermediate between that described by Daubney and Nickolls and that finally adopted. Separations not complete in initial stage
1	Brain	25	102	} Five successive experiments to check final procedure as described on pp. 000. All emulsions reduced and all separations clean.
1	Brain	25	105	
1	Brain	25	103	
0	Brain	25	12	
0	Brain	10	0	

ties from the alkaloid. Panzer (17) demonstrated that the Stas-Otto process failed to remove substances which gave the common color reactions of the alkaloids, even after special efforts at purification. This was particularly true in the case of brain tissue. The degree of error, however, may be estimated by control experiments. The result of such an experiment is shown in table 4. Twenty-five grams of brain, containing no alkaloid, introduced a background absorption of 12 per cent (fig. 5). Smaller amounts of tissue, 10 grams for example, such as were desired in our studies, gave no spurious

<sup>4</sup> The cause of the parallel shifts in the calibration curves shown in figure 5, which were made at successively later dates, and their cessation, is in doubt. It may be attributed either to changes in the equilibrium temperature of the source over a long period of time or to gradual changes in the aging of a freshly opened bottle of Rodinal developer. It serves to emphasize the fact that calibration curves of this kind must be checked frequently.



absorption. If 12 per cent were subtracted from the yields obtained in the *in vitro* experiments (table 4) the corrected yields were about 90 per cent or 0.9 mgm. Although the impurities which remained were quantitatively many times the amount of cocaine used in these experiments, they remained either insoluble in the final solvent or produced no serious absorption in the region of the spectrum involved by cocaine absorption. The error of the method was estimated to be within 10 per cent. The step-by-step elimination of errors in the method by numerous experiments not included in this report and the consistency of results obtained with the final procedure, make us confident that this estimate of error is conservative.

*In vivo* experiments (to be reported) using this method are in progress. The relative distribution of cocaine in brain, cerebrospinal fluid, and blood has been measured in a series of experiments in which cocaine hydrochloride was

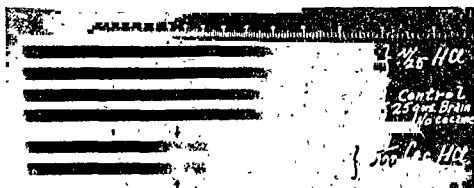


FIG. 5. DUPLICATE SPECTROGRAMS OF THE SOLVENT, OF A 0.1 PER CENT SOLUTION OF COCAINE HYDROCHLORIDE AND OF A SOLUTION OBTAINED BY EXTRACTING 25 GRAMS OF BRAIN TISSUE ACCORDING TO THE PROCEDURE DESCRIBED IN THE TEXT

Note the lack of absorption in the spectrogram of the control in the region of the absorption bands used for the measurement of cocaine hydrochloride—indicated by the arrows.

injected intravenously. Consistent results have been obtained and in one case as little as 0.05 mgm of cocaine was measured in 12 gm. of brain.

#### SUMMARY

By means of a spectrochemical method which has high sensitivity and good reliability for measuring one milligram or less of cocaine, a critical study of the details of some of the reported methods of analysis of alkaloids has been made. The sources of errors in these methods were analyzed and a technique developed which has given high yields of cocaine from tissues *in vitro*. Analyzing for 1 mgm. of cocaine hydrochloride in 25 grams of tissue, the technique finally adopted gave consistent yields, when corrected, of approximately 90 per cent, with an estimated error of 10 per cent. The method has been found applicable to quantities as small as 0.05 mgm. of cocaine in experiments *in vivo*. Isolation and measurement required from seven to nine hours.



We are indebted to Professor D. M. Greenberg of the Division of Biochemistry for certain laboratory facilities and for making many valuable suggestions in the course of this investigation.

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# AN ANALYSIS OF THE ACTION OF BULBOCAPNINE IN SPINAL ANIMALS

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Received for publication August 4, 1941

Studies by Baruk and de Jong (1), Kraus and de Jong (2), Girndt and Schaltenbrand (3) on the action of bulboCAPNINE in a variety of vertebrates indicate that the drug induces a cataleptic state,—a resultant, in part at least, of the action of the drug through the cerebral cortex. Further experiments, particularly those of Schaltenbrand (4, 5, 6), demonstrate that other parts of the nervous system are affected by the drug. Schaltenbrand also noted a strychnine-like action with subconvulsive doses of bulboCAPNINE. Ferraro and Barrera (7), in a thorough re-analysis of the actions of bulboCAPNINE on cats and monkeys, concluded that the drug is a diffusely acting poison, affecting not only the central nervous system but peripheral nervous structures and perhaps even muscular structures themselves. With exception of Schaltenbrand's experiments and those of Ferraro and Barrera, no detailed observations have been made on the effect of bulboCAPNINE in the spinal animal. In the present experiments we have obtained graphic records of the flexor and extensor muscular tone in the legs of spinal cats under bulboCAPNINE. We believe that this method of recording furnishes a more reliable measure of the effect of bulboCAPNINE on muscle tone than methods previously employed, and that the results prove that an intact reflex arc is necessary for the manifestations of tone attributable to bulboCAPNINE in the spinal animal.

**MATERIALS AND METHODS.** BulboCAPNINE phosphate (Merck) was used in these experiments. It was given in "moderate" doses, approximately 30 mgm. per kilogram. Fifteen cats were used; the cord of each animal was cut between the 10th and 12th thoracic segment. All operations were done under nembutal (30 mgm. per kilogram). The dorsal roots were sectioned on one side in three, and on both sides in one animal. The entire cord below the point of section was removed in one animal. Experiments were made acutely (within 36 hours after cord section) and chronically (48 hours or more after section). The operations were done aseptically and 24 hours lapsed from the time of operation before the injection of bulboCAPNINE in the acute spinal preparations.

The method used for recording changes in tone in the legs was a modification of that described by Briscoe (8) for studying tone in the legs of decerebrate animals. The

<sup>1</sup> This work was begun while one of us was a National Research Council Fellow in the Department of Anatomy at the Johns Hopkins University Medical School.



cats were suspended prone in a cradle, legs free, and a light cord, attached to the paw of the extremity being studied, was passed through a pulley to a writing lever. The lever recorded the movements of the leg on a moving kymograph drum. For a determination of flexor tone the leg was flexed to a given height (upper horizontal base line on the tracings) and then was allowed to fall, pulled down by gravity. Extensor tone was measured in a similar manner by allowing the extended leg to fall into a flexed position while the cat was lying in the cradle on its back. In later experiments, so that determination of flexor and extensor tone could be made more rapidly and without altering the prone position of the animal, a weighted cord was passed over a pulley and attached to the leg. The weight was sufficient to flex the extended leg of the spinal animal at a rate similar to that obtained by the first method. The leg was allowed to flex from a given point of extension (lower horizontal base line) for each determination of the extensor tone.

The tone in the legs was expressed by the distance the leg fell and the amount of oscillation occurring at the end of the fall. By comparing a series of records in a given experiment, it was possible to determine any fluctuation in the flexor or extensor resistance with relative accuracy.

**RESULTS.** *Effect of bulbocapnine in spinal animals, acute and chronic.* The effects of bulbocapnine in moderate doses (30 mgm. per kilogram body weight) observed in spinal animals anterior to the level of the spinal cord section have been entirely similar to those described by Ferraro and Barrera (7) and Ingram, Barris and Ranson (9) in intact animals. Significant differences in the effects of bulbocapnine on the hind quarters of normal and spinal animals were noted, however. The hind legs of the spinal animals were unaffected by bulbocapnine during the first 24 to 36 hours after the cord was cut. Following this period, however, the drug led to an increase in resistance to passive movements of the hind legs, with the legs held in relative extension. As a rule urination and defecation occurred within 5 to 10 minutes after the injection in the chronic animals.

In old chronic spinal preparations subconvulsive doses of bulbocapnine, instead of producing a constant plastic state in the caudal portion of the animal, brought about "mass reflex" activity following the slightest of cutaneous or proprioceptive stimuli (at times the stimulus was not self-evident). The response consisted of bilateral flexion of the hind legs often accompanied by micturition and defecation. Frequently jerky rhythmic movements of the limbs followed the flexor movements. When these subsided the movements could be elicited again by a similar stimulus. Movements of this sort often occurred in the deafferented limb in the spinal animals with the dorsal roots cut on one side.

The period of flaccidity or "spinal shock" persisted from 24 to 36 hours in the cats. The type of response obtained during this period is shown in figure 1, A and B; the former representing the 'flexor fall' and the latter the 'extensor rise.' The first portion of each record represents the passive adjustment of the limb to the base line, the second the fall of the leg under the influence of gravity, and finally the reaction of the leg at the end of the fall. In the spinal



animal it is clear that there is a slight rebound expressed by the short secondary rise. The responses obtained in *E* and *F* of figure 1 were recorded 51 and 54 minutes after the subcutaneous administrations of 20 mgm. of bulbocapnine per kilogram. The similarity of *E* and *F* to *A* and *B* led us to conclude that the drug has no action in the caudal segments of the body during the period of flaccidity (spinal shock).

In chronic preparations the resistance to passive movements, particularly to flexion, was marked, and reflex activity was easily elicited. In figure 2, *A* and *B* illustrate the responses obtained prior to bulbocapnine 15 days after

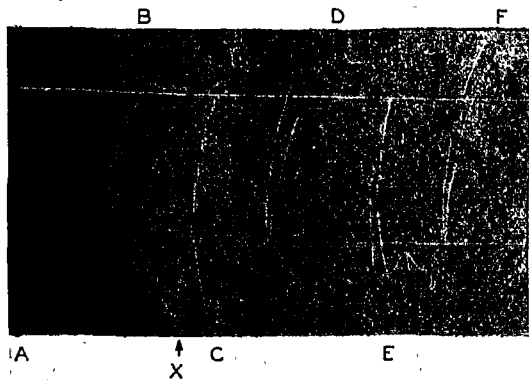


FIG. 1. ACUTE SPINAL PREPARATIONS 24 HOURS AFTER OPERATION

*A* and *B*, normal flexor fall  
nine, subcutaneously at 9:20.  
*E* and *F*, flexor fall and extens  
change in the cat's position in

the cord was cut at the level of the 10th thoracic vertebra. The alteration in the tone 14 and 15 minutes after the injection of 30 mgm. bulbocapnine per kilogram body weight is shown in the tracing at *C* and *D*, *E* and *F*. Twenty-one hours later the tracings were normal again.

*Effect of bulbocapnine on spinal animals with unilateral dorsal root section below the level of cord transection.* Four experiments of this sort show that bulbocapnine induced an alteration in the extensor activity of the hind leg, but this was associated with little or no change in the flexors. In one other experiment in which the contralateral hind leg was tested a similar result was observed. We cannot state that the cutting of the dorsal roots on one side in



the spinal preparations leads to a complete loss of tone in the flexor muscles in the presence of bulbocapnine, but certainly the operation caused a great reduction of tone in these muscles (fig. 3), as compared with the spinal animal with sensory nerves intact.

*Effect of bulbocapnine in spinal animals with dorsal roots cut bilaterally.* In one cat the lumbosacral dorsal roots were cut two days before bulbocapnine was given; the cord was cut several days before at T12. The records showed no significant change. Eleven days later the experiment was repeated and

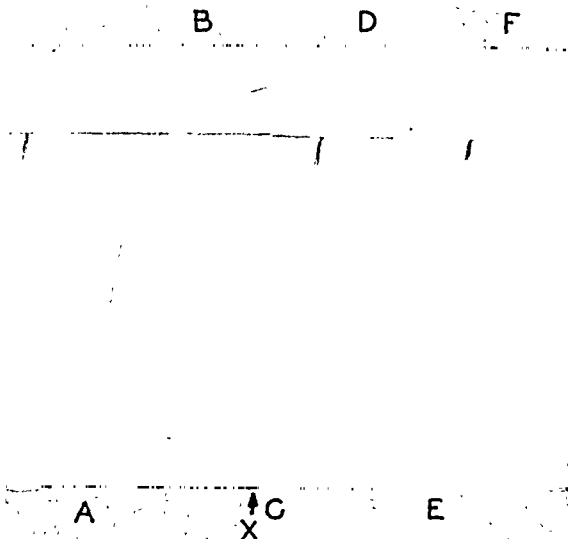


FIG. 2. CHRONIC SPINAL PREPARATION (15 DAYS SINCE OPERATION)

A and B, normal flexor fall and extensor rise, respectively. X, 70 mgm. bulbocapnine, subcutaneously at 1:55. C and D, extensor rise and flexor fall at 2:09 and 2:10. E and F, extensor rise and flexor fall at 2:22 and 2:21:30. Examination of the record shows slight decrease in tone (?) in flexors at 2:21:30 as compared with that at 2:10; this is partly due to a shift in the animal's position in the cradle. Tone is obviously increased in both flexors and extensors over the state prior to the injection of bulbocapnine.

no change in the responses was obtained following the administration of bulbocapnine (fig. 4). In two other animals similar results were noted.

*Effect of bulbocapnine following the complete removal of the lumbar and sacral spinal cord.* In one animal the spinal cord was removed from the level of the 10th thoracic vertebra downward. Twenty-four hours later records were obtained of the 'fall' and 'rise' of one hind leg before and after bulbocapnine (30 mgm. per kilogram body weight). Records were also obtained the next day, after the animal had recovered from the catalepsy exhibited the day before anterior to level of cord section. An examination of all the records



showed that there was no significant change attributable to the direct effect of bulbocapnine on the muscles of the hind limbs.

**DISCUSSION.** This study emphasizes the fact that bulbocapnine may produce an effect in the spinal cat as well as in one with an intact nervous system. The manifestations of the action of the drug in the caudal portion of the spinal animal differ, however, from those observed in the same part of a normal animal. This is quite evident in that extensor tone predominates in the spinal cat whereas a flexed posture of the hind legs is characteristic of the catalepsy in the intact animal. As Ferraro and Barrera point out, the effect of bulbo-

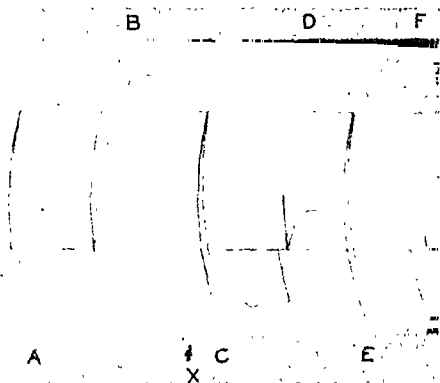


FIG. 3. CHRONIC SPINAL PREPARATION (16 DAYS) WITH IPSILATERAL DORSAL ROOT SECTION BELOW SPINAL SECTION (2 DAYS)

A and B, normal flexor fall and extensor rise, respectively. X, 100 mgm. bulbocapnine. Tone so great bulbocapnine

capnine intoxication is the result of the action of the drug on the entire nervous system, and it is equally evident that some portions of the nervous system may overshadow and alter the effects of another portion, or the combined result of the influences of the two may be entirely different from that of either or their expected summed result.

The explanation of the phenomena observed in the records of the 'rise' and 'fall' in all the experiments is not completely clear. In chronic spinal animals, however, with intact sensory nerves to the hind legs, intrinsic reflex activity (ipsilateral) is probably quite significant. The stretch reflex in the extensors



operates to shorten the 'rise' in the records, and the 'pluck' reflex of Asayama (10) operates similarly in the flexors. In the acute spinal preparations no reflex activity was observed. Our records from acute spinal preparations are not essentially different from those obtained from animals with bilateral dorsal root section below the level of cord transection or from animals with lumbo-lumbar cord removed. This indicates a lack of effect of bulbo-capsine on the muscle directly as far as its state of contraction is shown by the method em-

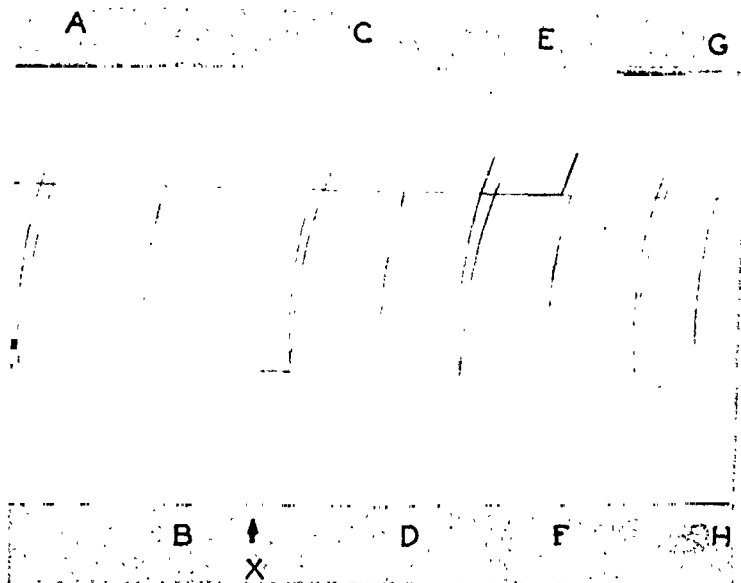


FIG. 4. LUMBO-SACRAL DORSAL ROOTS CUT BILATERALLY 13 DAYS BEFORE; THE CORD WAS CUT PRIOR TO THE DORSAL ROOT SECTIONING

A and B, normal extensor rise and flexor fall, respectively. X, 100 mgm. bulbo-capsine injected, subcutaneously at 2:00. C and D, extensor rise and flexor fall at 2:09. E and F, extensor rise and flexor fall at 2:50. G and H, extensor rise and flexor fall at 2:59. The effect in F and H and E and G are due to shifts in the animal's position in the idle. No significant changes are present in the records.

ployed. We are not familiar with any work dealing with the effect of bulbo-capsine on the nerve-muscle preparation.

It is evident that other factors than intrinsic reflex activity contribute to the records obtained from the spinal animals with ipsilateral dorsal root section below the level of the spinal section. The presence of an effect in the extensor muscles of the deafferented limb may indicate that tone is present in the limb. That tone may exist in the deafferented leg is supported by the observation on a decerebrate animal (with dorsal roots cut to one fore leg) in which the usual tonic neck reflexes of Magnus and de Kleijn were demonstrated (unpublished work) and by Wilson (11) who states (p. 390), "motor ac-



tivities . . . essentially postural . . . can be obtained by appropriate stimulation" of the mesencephalon . . . and are "maintained for many seconds, or even minutes, in limbs all the posterior roots of which have been cut months before." In the records on spinal animals with ipsilateral dorsal root section the 'tone' was maintained at least for a matter of seconds with only slight 'give' in the extensors, and is evidence of a crossed extensor reaction. It is of interest that in none of our records of the extensor activity, even in the chronic spinal animals with sensory nerves intact, was recruitment obviously present, though the 'give' in the extensors was less than in the flexor muscles.

From our results it would appear either that bulbocapnine affects primarily the afferent portion of the nervous system, (i.e., the sensory nerve fibers or their endings) or some synapse in the cord. If the latter is true, the effect is dependent on incoming impulses which play on these centers, for the drug itself appears not to initiate efferent impulses in the absence of afferent stimuli.

The absence of demonstrable evidence of tone in the records of the flexor 'fall' in the ipsilateral extremity (and in one case, contralateral) of unilateral dorsal root preparations may be associated with the greater tonic activity in the antigravity muscles. Similar experiments on the sloth would be of interest in this connection since Langworthy (12) has shown that the flexors, the antigravity muscles in this animal, are chiefly affected by decerebration and produce typically a flexor rigidity.

Schaltenbrand's so-called strychnine effect appears to be represented in the spinal animal by the mass reflex and jerky rhythmic activity that is present in chronic preparations. Convulsions are elicitable with bulbocapnine in normal animals (4), but larger doses (70 mgm. per kilogram) are necessary than were used in our experiments. In any case, the forward end of these spinal animals never showed these convulsive movements but manifested the typical catalepsy. The mechanism of the "strychnine-like" effect in the spinal animals under bulbocapnine is probably not the same as with strychnine, i.e., poisoning of sensory and/or motor cells or synapses (Dusser de Barenne (13)), but as indicated by the present experiments, is probably the effect of some altered mechanism in peripheral sensory nerve fibers or structures directly associated with them functionally (ending or synapse).

#### SUMMARY AND CONCLUSIONS

On the neuro-muscular activity of spinal cats bulbocapnine produced definite effects characterized by an increased tone in the flexor and extensor muscles of the hind limbs. The extensors were primarily affected. In the unilaterally deafferented spinal animal bulbocapnine caused a resistance to passive flexion but little or no resistance to passive extension. No significant changes in the resistance to passive movements of the hind legs were elicited with bulbocapnine in the animal with spinal cord removed, in the acute spinal animal, or in the spinal animal with bilateral dorsal root section below the



cord lesion. The effect of bulbocapnine on the musculature of the hind legs of the spinal animal was dependent on the presence of some sensory fibers of the isolated spinal cord segments. A quick and accurate method for recording changes in the flexor and extensor tone of a limb with intact innervation and muscle attachment is described.

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# ON THE MODE OF ACTION OF THE SULFONAMIDES<sup>1</sup>

## II. THE SPECIFIC ANTAGONISM BETWEEN METHIONINE AND THE SULFONAMIDES IN *ESCHERICHIA COLI*<sup>2</sup>

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Received for publication August 4, 1941

In the preceding paper it was demonstrated that the primary action of the sulfonamides is upon the synthetic rather than the oxidative mechanisms of *E. coli*. From the relation between inhibition (of growth) and drug concentration we deduced that a number of such synthetic systems are involved. The hypothesis was therefore elaborated that sulfonamides, either directly or through competition with *p*-amino-benzoic acid (1), interfere with the synthesis of several materials necessary for bacterial growth. Growth and multiplication slow and finally cease as the supply of these substances fails. Conversely, we would expect that the addition of these materials to the medium should antagonize the action of sulfonamide. With this in mind, we have tested all the commonly available amino acids and some other substances concerned with bacterial nutrition. Of these, only methionine and *p*-amino-benzoic acid showed antagonistic activity under the conditions of our survey.

The methods and materials employed, including the strain of *E. coli*, were the same as previously described (2). This strain, which can be grown in a medium of inorganic salts and glucose, is well suited for such a study since its metabolism involves a maximal number of synthetic reactions. As explained previously, we measure the rate of growth by the velocity constant of multiplication. When this is calculated from data involving the time for a small inoculum to reach a density of 132 million cells per cubic centimeter, it represents a mean value for this entire period, and is designated by the subscript *m* as *K<sub>m</sub>*. *K<sub>m</sub>* is approximately equal to the fraction of the total population dividing each minute.

### EXPERIMENTAL

#### *Test for antagonistic activity*

Various neutralized substances were added to 8 cc. of medium SG (2) (inorganic salts and glucose), the final concentration being  $10^{-2}$  or  $10^{-4}$  M.

<sup>1</sup> Aided by a grant from the Duke University Research Council.

<sup>2</sup> A preliminary report of some of this work was given at the American Physiological Society meeting, Chicago, April 17, 1941.



The medium was inoculated with about 150 organisms and incubated at 37°C. The anti-sulfonamide activity of the test substance was determined by its ability to cause visible growth (132 million organisms per cc. measured photometrically) in the presence of sulfanilamide concentrations ( $10^{-3}$  and  $2 \times 10^{-4}$  M) known to prevent it in the controls. Experiments were discontinued at 90 hours when the larger sulfanilamide concentration was used, and at 55 hours for the smaller.

The following amino acids were tested: *dl* alanine,  $\beta$  alanine, methyl  $\beta$  alanine, *dl* allothreonine,<sup>3</sup> *dl*  $\alpha$ -amino butyric acid, *dl*  $\alpha$ -amino isobutyric acid, *dl*  $\alpha$ -amino  $\alpha$ -methyl butyric acid, *dl*  $\alpha$ -amino caprylic acid, *dl*  $\alpha$ -amino heptylic acid, *l*(+) arginine, *l* asparagine, *dl* aspartic acid, *l*(-) cysteine, *l*(-) cystine, *dl* ethionine, *dl* glutamic acid, *l*(+) glutamine, glycine, *dl* histidine, *dl* homocystine, *dl*-iodotyrosine, *dl* isoleucine, *dl* leucine, *l* lysine, *dl* methionine, *dl* norleucine, *dl* norvaline, *l*(-) oxyproline, ornithine, *dl* phenyl alanine, *dl* proline, *dl* serine, *dl* threonine,<sup>3</sup> *l*(-) tyrosine, *l*(-) tryptophane, *dl* valine. Other substances were: adenine, guanine, 5 amino uracil, xanthine, hypoxanthine,

TABLE 1

*Effect of amino acids upon growth in presence and absence of sulfanilamide*

AMINO ACID	TIME (IN HOURS) TO REACH VISIBLE GROWTH (132 MILLION PER CC.)	
	No sulfanilamide	$2 \times 10^{-4}$ M sulfanilamide
<i>l</i> -glutamic $10^{-3}$ M.....	17	>50
-tyrosine $10^{-3}$ M.....	16	>50
-cystine $10^{-3}$ M.....	15	>50
l-cystine $10^{-3}$ M.....	14	>50
All the above together, each at $10^{-3}$ M.....	12.5	>50
<i>l</i> -methionine $10^{-4}$ M.....	15.5	24.5

lloxan, alloxantin, parabanic acid, allantoin, propionic acid, butyric acid, valeric acid, aproic acid,  $\epsilon$ -amino caproic acid, heptylic acid, putrescine, cadaverine, choline, cholesterol, creatine, creatinine, thiamine, riboflavin, nicotinic acid, 2 amino nicotinic acid, 5 amino nicotinic acid, 6 amino nicotinic acid, nicotinamide, cozymase ( $10^{-4}$  M), denylic acid, pyridoxin ( $B_6$ ), pantothenic acid, *d* glucosamine, glutathione, 2-methyl, 4-naphthohydroquinone diacetate.

Of all the substances tried only methionine and *p*-aminobenzoic acid gave positive results. The effect of methionine cannot be attributed to a non-specific stimulation of growth for, as seen in table 1, other substances can stimulate growth more than methionine in the absence of drug, but have no effect on the sulfonamide inhibition under the conditions of the experiment. This is particularly true for the combination of cystine, tyrosine and glutamic acid.

<sup>3</sup> We are indebted to Dr. M. Carter for a supply of *dl* threonine and *dl* allothreonine.



*Concentration of methionine*

The data of a typical experiment illustrating the effects on growth when the *dl*-methionine and sulfonamide concentrations were altered are shown in table 2. It is seen that the net result depends upon the concentrations of both agents. At a sulfanilamide concentration of  $5 \times 10^{-5}$  M,  $K_m$  is 68 per cent of the control; the addition of  $10^{-7}$  methionine increases this to 81 per cent, and of  $10^{-5}$  to 94 per cent. Thus, increasing the methionine concentration decreases the inhibition, in this case almost completely. However, at a sulfanilamide concentration of  $10^{-3}$  M, the addition of  $10^{-5}$  M methionine increases  $K_m$  from less than 13 to 17 per cent of the control, and further in-

TABLE 2  
*dl*-Methionine vs. sulfonamide

<i>dl</i> -METHIONINE	SULFANILAMIDE CONCENTRATION, PER CENT CONTROL $K_m$ *	SULFAPYRIDINE CONCENTRATION, PER CENT CONTROL $K_m$	SULFATHIAZOLE CONCENTRATION, PER CENT CONTROL $K_m$
<i>molar</i>	<i>molar</i>	<i>molar</i>	<i>molar</i>
0	$5 \times 10^{-5}$ 68	$2 \times 10^{-5}$ 71	$5 \times 10^{-7}$ 48
$10^{-7}$	81	79	71
$10^{-5}$	94	76	88
$10^{-3}$	94	76	88
0	$2 \times 10^{-4}$ 13	$1 \times 10^{-5}$ 17	$2.5 \times 10^{-6}$ <13
$10^{-7}$	30	18	18
$10^{-5}$	60	55	43
$10^{-3}$	56	50	43
0	$1 \times 10^{-3}$ <13	$5 \times 10^{-4}$ <13	$1 \times 10^{-6}$ <13
$10^{-7}$	<13	<13	<13
$10^{-5}$	17	<13	<13
$10^{-3}$	13	<13	<13

\*  $K_m$  is the mean value of the growth constant, and is inversely proportional to the time required for the inoculum of about 150 organisms to reach a density of 132 million per cubic centimeter.

creases in methionine concentration would not raise this value. Further study of the table shows that for every sulfonamide concentration there is a ceiling which limits the maximum antagonistic action of methionine. Below this ceiling, increasing the methionine concentration will decrease the inhibition. At low sulfonamide concentrations the ceiling is high—methionine when present in adequate concentration can then be almost a perfect antagonist. At high sulfonamide concentrations the ceiling is low, and methionine, no matter what its concentration, has little or no effect. In any case, the maximum effect of methionine is reached at a concentration between  $10^{-5}$  and  $10^{-4}$  M, there being a decreased activity at both higher and lower concen-



trations. It is also seen that methionine is less active against sulfathiazole and sulfapyridine than against sulfanilamide.

At this juncture it is useful to recall the theory advanced in the previous paper (2); that the number of loci affected directly or indirectly by the sulfonamides increases with the concentration of drug. On this basis we suppose that the synthesis of methionine is one of the first to be eliminated and therefore methionine is a good antagonist at low drug concentrations. Methionine,

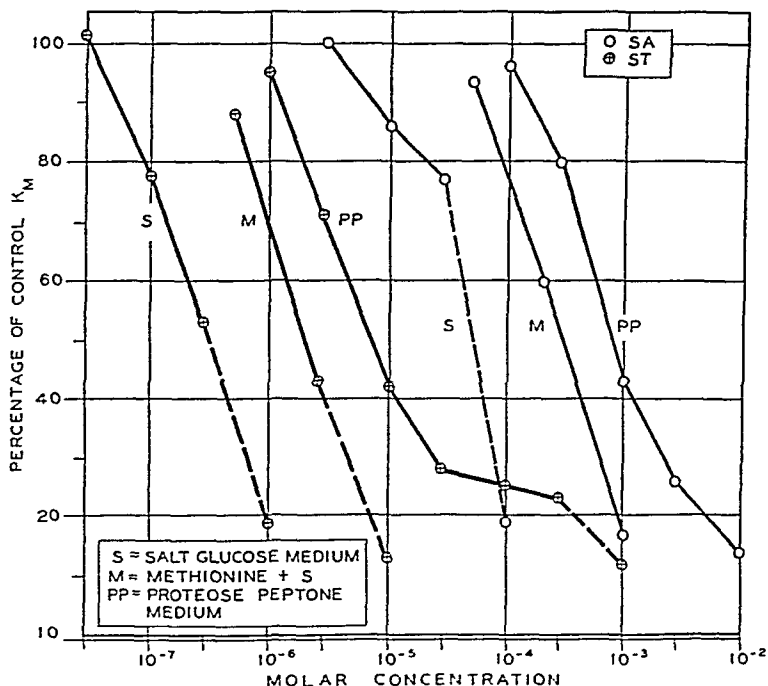


FIG. 1. THE EFFECT OF DRUG CONCENTRATION UPON THE GROWTH OF *E. COLI* IN SALT-GLUCOSE MEDIUM, SALT-GLUCOSE MEDIUM CONTAINING  $10^{-5}$  M *dl*-METHIONINE AND PROTEOSE-PEPTONE-FUMARATE-GLUCOSE MEDIUM

The growth constant  $K_M$  (as percentage of control) is plotted against drug concentration

however, becomes less effective at higher drug concentrations as other reactions upon which growth depends are inhibited.

In the richer PPFG medium (proteose-peptone, fumarate, glucose) methionine fails to show any effect on the sulfonamide inhibition. This is to be expected for the medium already has sufficient antagonistic activity to raise the effective concentrations of sulfanilamide above those at which methionine is still active. Furthermore, optimal amounts of methionine probably are present in the PPFG medium, since if the dry proteose-peptone (Difco) con-



tained as little as 0.01 per cent methionine the final medium would have a concentration of  $10^{-5}$  M.

It is instructive to compare methionine, proteose-peptone and *p*-amino-benzoic acid in their antagonistic activity to the sulfonamides. The curves in figure 1 relate  $K_m$  to sulfanilamide (open circles) and to sulfathiazole concentration (crossed circles) in three different media—SG, SG containing  $10^{-5}$  M *dl*-methionine, and PPFG.

Methionine shifts the entire curve to the right along the concentration axis but does not change its shape. Proteose-peptone shifts the curve still farther to the right and changes its shape by putting a plateau at the bottom of the sulfathiazole curve (and also of the sulfapyridine and sulfadiazine, but not the sulfanilamide curves).

Analysis of these curves, as illustrated in figure 2 shows the presence of at least three substances capable of antagonizing sulfonamides, i.e., shifting the curves to the right so that the same concentration of drug produces a smaller

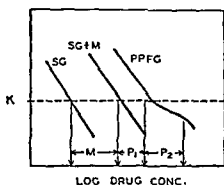


FIG. 2. FIGURE 1 ANALYZED ACCORDING TO THE TEXT

inhibition in the growth constant  $K_m$ . The shift of the concentration curve in SG to that in SG plus methionine is labeled  $M$  and defines the maximum methionine effect. The second shift labeled  $P_1$  is due to some substance in PPFG which antagonizes in the lower concentration range. The third shift,  $P_2$ , is obtained in the higher concentration range and only with sulfathiazole, sulfapyridine and sulfadiazine.

We suppose that each of these three well-defined shifts in the concentration curve is due to a specific antagonist, and that each antagonist must interact at a different point in the inhibited bacterial metabolism. By this empirical means there are defined three inhibited loci:

- That inhibited by all drugs, but for which methionine can compensate.
- That inhibited by all drugs, but for which methionine cannot compensate. Something in proteose-peptone can ( $P_1$ ).
- That only inhibited by sulfapyridine, sulfathiazole, and sulfadiazine, and for which something in proteose-peptone can compensate ( $P_2$ ).



ocus (a) probably is the synthesis of methionine; the nature of (b) and (c) cannot be guessed.

The question arises whether shift  $P_1$  or  $P_2$  might be due to the presence of  $p$ -aminobenzoic acid in the proteose-peptone. To study this, we added  $p$ -aminobenzoic acid to SG so that the final concentration varied from  $10^{-9}$  to  $10^{-2}$  M in steps of 10. At each level we varied the concentration of ulfathiazole. As the concentration of  $p$ -aminobenzoic acid rose from 0 to  $10^{-4}$ , the curve relating  $K_m$  to drug concentration (see fig. 2) shifted progressively to the right, but no plateau was formed as occurs in PPF<sub>G</sub>. Since no plateau was induced, shift  $P_2$  cannot be due to  $p$ -aminobenzoic acid, though it might be. At  $10^{-3}$  and  $10^{-2}$  M  $p$ -aminobenzoic acid, the curve tended to move back to the left, these higher concentrations being less effective than  $10^{-4}$  M; in the absence of sulfonamide, they are inhibitory in themselves.

From this analysis it follows that there are at least three distinct antagonists,  $p$ -aminobenzoic acid, methionine, and at least one more substance found in proteose-peptone. Of these, methionine is the least potent, since it is effective only at the lowest concentrations of the sulfonamides.  $p$ -aminobenzoic acid seems to be the most potent and the most general in its action. The third antagonist, defined by shift  $P_2$ , is rather specialized since it is not concerned with sulfanilamide, but only the other three drugs, and at their highest concentrations.

#### *Inhibition and antagonism as a function of time*

The action of methionine as revealed by the plate counting technique is shown in figure 3, where the log number of organisms in SG is plotted against time.

The inhibition caused by  $2 \times 10^{-4}$  M sulfanilamide becomes apparent between the 50th and 100th minute, and by 250 minutes has approximately reached a maximum. The presence of  $10^{-5}$  M *dl*-methionine greatly reduces this inhibition, although it does not appreciably change the time of onset. It is seen that methionine does not alter the rate of control growth, though it does shorten the induction period.

For a closer analysis of the relationships involved we have studied the change in the growth constant with time as shown in figure 4. These data were obtained by using serial dilutions of inoculum and measuring the time for each to reach a photometric endpoint of 132 million organisms per cubic centimeter. The number of divisions required to reach the endpoint is plotted against the time. Since

$$N = N_0 \times 2^D$$

where  $D$  is the number of divisions,  $N_0$  the inoculum and  $N$  the number present at time  $T$ ,

$$K_{ad} = 0.69 (D/T)$$



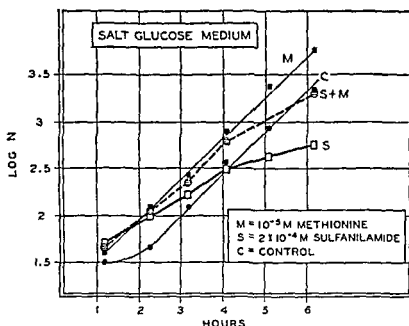


FIG. 3. THE COURSE OF SULFANILAMIDE INHIBITION DETERMINED BY VIABLE COUNTS  
Logarithm of bacterial population is plotted against time

C, control growth in a salt-glucose medium; M, growth in a salt-glucose medium containing  $2 \times 10^{-4}$  M methionine

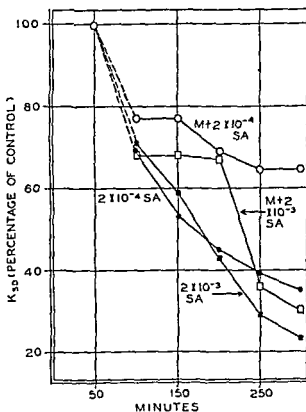


FIG. 4. THE COURSE OF SULFANILAMIDE INHIBITION IN SALT GLUCOSE MEDIUM WITH AND WITHOUT  $10^{-5}$  M dl-METHIONINE

The growth constant  $K_{30}$  (as percentage of the control without drug or methionine) is plotted against time



or 0.69 times the slope of the curve relating number of divisions to time. Provided that the percentage of viable organisms is constant (as we have previously shown), that there is no latent period (a large inoculum from a rapidly growing culture is used), and that the medium is not exhausted,  $K_{ad}$  has the same value as  $K$  determined by plate counts.

Three types of medium were employed, SG, SG plus  $10^{-5}$  M methionine, and PPF<sub>G</sub>. In each case, the inhibitions appeared only after at least 50 minutes. In SG, the curves for  $2 \times 10^{-3}$  and  $2 \times 10^{-4}$  are relatively smooth and follow the same course during the first 200 minutes. Thereafter the higher concentration of drug produces a greater inhibition than the lower. In the presence of  $10^{-5}$  M methionine each curve has a plateau between 100 and 200 minutes during which the inhibition does not increase. Thereafter at the lower sulfanilamide concentration the inhibition increases somewhat but becomes constant so that growth continues indefinitely although somewhat slower than normally. At the higher sulfanilamide concentration, the inhibition then increases rapidly and closely approaches the control. A similar situation holds for PPF<sub>G</sub> where much higher concentrations of sulfanilamide ( $2 \times 10^{-3}$  and  $2 \times 10^{-2}$  M) are necessary because of the antagonists in the medium.

These findings may be explained by the hypothesis already advanced: that an increasing number of loci are affected as the drug concentration is raised, and also as contact of the growing organism with the drug is prolonged. The presence of methionine compensates for one of the first loci to be inhibited. This is seen in figure 4 where the inhibition (i.e., decline in  $K$ ) begins, but is then arrested. This arrest is practically complete at 200 minutes when sulfanilamide concentration is  $2 \times 10^{-4}$  M, after which growth continues at a steady rate. At  $2 \times 10^{-3}$  M, however, the arrest is only temporary, and the inhibition rapidly increases after the 200 minute, when obviously other loci, for which methionine cannot compensate, become affected.

At low drug concentrations (e.g.,  $2 \times 10^{-4}$  M sulfanilamide),  $10^{-5}$  M methionine in SG permits growth indefinitely, without the acquisition of resistance, since after 15 subcultures (about 360 divisions) inocula failed to grow out within 96 hours in SG containing  $2 \times 10^{-4}$  M sulfanilamide but no methionine.

Methionine is effective even when added some hours after sulfanilamide, though it is not as efficient or as rapidly acting as *p*-aminobenzoic acid. Table 3 shows the results of an experiment in which 8 cc. of SG medium containing  $2 \times 10^{-4}$  or  $2 \times 10^{-3}$  M sulfanilamide was inoculated with 350 organisms and incubated at 37°. At various intervals, *p*-aminobenzoic acid or *dl*-methionine (final concentration of  $10^{-5}$  M) was added and the time to reach a density of 132 million organisms per cubic centimeter was determined. It is seen that methionine is active even when added 14 hours after the sulfanil-



amide. *P*-aminobenzoic acid works more rapidly and is efficacious at high concentrations of sulfonamide.

### *Specificity of methionine*

The chemical specificity of methionine action was determined by comparing the action of the two isomers and of other closely related substances. Four different commercial preparations of *dl*-methionine gave identical results. In figure 5, the effects of the two isomers from  $5 \times 10^{-2}$  to  $10^{-3}$  M are compared in the presence and absence of  $2 \times 10^{-4}$  M sulfanilamide.<sup>4</sup>  $K_m$  is plotted as the percentage of the control value determined in the absence of

TABLE 3

Eight cubic centimeters of SG containing SA were inoculated with 350 organisms at zero time; antagonist was added at various intervals thereafter, and the time to reach a density of 132 million per cubic centimeter was determined.

ANTAGONIST 10 <sup>-3</sup> M	SA	TIME AT WHICH ANTAGONIST ADDED	TIME AT WHICH ENDPOINT REACHED	DIFFERENCE
	molar	hours	hours	hours
<i>dl</i> -Methionine	$2 \times 10^{-4}$	2	19	17
		4	23	19
		6	30	24
		8	34	26
		14	41	27
	$2 \times 10^{-3}$	0	Not reached in 150 hours	
<i>p</i> -Aminoben- zoic acid	$2 \times 10^{-4}$	0	19	19
		8	20	12
		16	27	11
	$2 \times 10^{-3}$	0	17	17
		4	19	15
		16	31	15

methionine or sulfanilamide. In the absence of the drug both isomers shorten the time to reach the endpoint, the *l*(-) form being 10-100 times as effective as the *d*(+) form. As previously explained, this effect is not upon the maximum rate of growth but upon the induction period which was rather long in this particular experiment. The effect shows up in  $K_m$  since this is the average growth constant throughout the entire period of growth until the endpoint is reached. In the presence of  $2 \times 10^{-4}$  M sulfanilamide, the *l*(-) form is about 10 times as active as the *d*(+) form. The small effect of the *d*-form might have been due to slight racemization during autoclaving and the course of the experiment.

<sup>4</sup> We are indebted to Dr. Madelyn Womack for a supply of *d* methionine ( $\alpha_D^{25} = +8.8$ ).



The action of some sulfur containing amino acids is shown in table 4. Methionine is the only one which antagonizes sulfanilamide. Ethionine actually inhibits the growth of the bacteria in the absence of sulfanilamide, reducing  $K_m$  to about 50 per cent of the control value at  $10^{-4}$  M concentration and to less than 20 per cent at  $10^{-3}$  M. It is of interest that this ethionine inhibition is not antagonized by *p*-aminobenzoic acid but is completely overcome by  $10^{-5}$  M methionine. Apparently methionine and ethionine compete for the same locus in the bacterium. Homocystine and cystine are without effect on sulfanilamide although they tend, like methionine, to shorten the induction period. Cystine and cysteine are broken down to hydrogen sulfide.

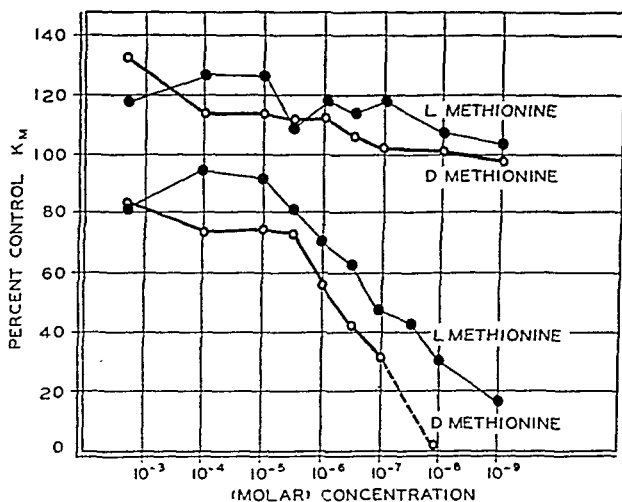


FIG. 5. THE EFFECT OF VARIOUS CONCENTRATIONS OF *d*- AND *l*-METHIONINE UPON THE GROWTH CONSTANT  $K_m$  (AS PERCENTAGE OF CONTROL) OF *E. COLI* IN SALT-GLUCOSE MEDIUM (UPPER CURVES) AND IN SALT-GLUCOSE MEDIUM CONTAINING  $2 \times 10^{-4}$  M SULFANILAMIDE (LOWER CURVES)

The importance of the amino group and of the sulfur in methionine is shown in table 5 by the failure of the straight chain fatty acid series and of their  $\alpha$ -amino acid derivatives to affect the sulfanilamide inhibition. It is interesting that the homologous series of  $\alpha$ -amino acids show inhibitory activity in SG, which begins with amino-butyric acid, reaches a maximum in amino-caproic acid (norleucine) and disappears with amino-heptylic acid. The specificity of these inhibitions is shown by the failure of acetylnorleucine, *N* (sulfanilyl) norleucine, *N* (sulfanilyl) norvaline and  $\alpha$ -hydroxycaproic acid to exhibit activity. Transfer of the amino group to the epsilon position (as in  $\epsilon$ -amino-caproic acid) or addition of an  $\epsilon$ -amino-group (as in lysine) also abolishes the inhibitory action. The activity of norvaline and particularly of norleucine,



like that of ethionine, is antagonized by methionine. None of these inhibitions can be demonstrated in PPFG which therefore contains antagonists for all (methionine).

TABLE 4

*The action of the sulfur-containing amino acids\**

ACID	FORMULA	ACTION	
		Growth	Sulfonamide inhibition
Ethionine†	$(C_2H_5)_2S \cdot (CH_2)_2 \cdot CH(NH_2) \cdot COOH$	Inhibits	Synergizes
Methionine.	$CH_3 \cdot S \cdot (CH_2)_2 \cdot CH(NH_2) \cdot COOH$	Shorten	Antagonizes
Homocystine...	$[S \cdot CH_2 \cdot CH_2 \cdot CH(NH_2)COOH]_2$	the in-	None
Cysteine	$HS \cdot CH_2 \cdot CH(NH_2)COOH$	duction	None
Cystine	$[S \cdot CH_2 \cdot CH(NH_2)COOH]_2$	period	None

\* We are indebted to Dr. Vincent duVigneaud for samples of ethionine and homocystine.

† Inhibitory action antagonized by methionine.

TABLE 5

*The action of straight chain acids tested at  $10^{-4}$  and  $10^{-3}$  M in medium SG*

ACID*	NUMBER OF CARBON ATOMS	EFFECT ON GROWTH
Propionic	3	None
Butyric	4	None
Valeric	5	None
Caproic	6	None
Heptylic	7	None
dl-alanine	3	None
dl- $\alpha$ -aminobutyric	4	Inhibits 30% at $10^{-3}$ M†
dl-norvaline	5	Inhibits 55% at $10^{-4}$ M‡
		Inhibits 85% at $10^{-3}$ M‡
dl-norleucine	6	Inhibits 85% at $10^{-4}$ M§
dl- $\alpha$ -aminoheptylic	7	None
dl- $\alpha$ -aminocaprylic	8	None
$\epsilon$ -aminocaproic	6	None
dl- $\alpha$ , $\epsilon$ -diaminocaproic (lysine)	6	None

\* None antagonize the action of sulfonamide.

† Not antagonized by methionine.

‡ Antagonized by methionine.

§ Most easily antagonized by methionine.

The importance of an unsubstituted amino group for the methionine antagonism of sulfanilamide is shown by the fact that *N* (sulfanilyl) methionine neither antagonizes sulfanilamide nor inhibits the growth of bacteria.



### *The metabolism of methionine*

The metabolism of methionine was studied in the Warburg apparatus in order to obtain a clue as to the mechanism underlying the antagonism. Suspensions of actively growing bacteria were centrifuged, washed once and resuspended in a buffer solution (WB) made up as follows: To 4 grams of NaCl, 1.5 gram of  $\text{KH}_2\text{PO}_4$ , 4.15 grams of  $\text{Na}_2\text{HPO}_4$ , add 1 liter distilled water; aerate and sterilize by boiling. Add 0.2 gram sterile  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ . This solution corresponds closely to medium SG but does not contain nitrogen or glucose. The pH is 7.2 and the molarity of the phosphate buffer is 0.04. The suspensions were placed in the Warburg vessels and the oxygen consumption measured in the usual fashion; the carbon dioxide production was measured by the direct and indirect Warburg methods. Bacteria suspended in WB show no growth and very little respiration. When 200 mgm. per cent of glucose is present, the oxygen consumption at 37°C. per billion cells per hour is 40 to 80 cmm. depending on the culture medium used previously. The R.Q. is approximately 1. This respiration slowly falls over the course of several hours.

The addition of *dl*-methionine to suspensions of bacteria in buffer, either with or without glucose, caused no additional oxygen uptake, carbon dioxide production and no detectable ammonia formation. Evidently methionine is not oxidized, decarboxylated or deaminated by the resting bacteria. The nitrogen of the methionine is not utilized for growth since the addition of both methionine and glucose to organisms suspended in WB did not result in growth. Comparable amounts of ammonia or arginine (and glucose) caused multiplication.

We may conclude, therefore, that the role of methionine in the metabolism of *E. coli* is neither to supply nitrogen nor energy for growth; this suggests that its role is a special one, probably concerned with anabolism. Three additional findings favor this suggestion: methionine preserves the respiration of the resting cell, decreases the induction period when growth begins, and stimulates the synthesis of respiratory enzymes.

### *Preservation of the respiration of the resting cell*

Bacteria grown in SG were centrifuged, and were washed and resuspended in WB (plus glucose) with and without *dl*-methionine ( $5 \times 10^{-4}$  M). The rates of oxygen consumption in microliters per hour per billion cells (which we shall call  $RO_2$ ) at the 25th, 115th and 265th minute were 51, 52, and 40 in the presence of methionine and 49, 44, and 33 in its absence.

### *The decrease in the induction period and stimulation of synthesis*

Actively growing organisms were centrifuged out of proteose-peptone medium, washed once with buffer salts containing glucose and resuspended in the latter solution at a density of  $3.5 \times 10^8$  organisms per cubic centimeter.



One portion of the suspension received 2 cc. of a *dl*-methionine solution such that the final concentration was  $5 \times 10^{-4}$  M; an equal portion of the original suspension received 2 cc. water. The two were incubated for four and one-half hours at  $37^\circ$ , during which time no growth (as measured photometrically) occurred. The suspensions were then separately centrifuged, washed and resuspended with and without methionine as before. Eight Warburg vessels were filled with 2.5 cc. of each suspension—four vessels of each set were used for the determination of oxygen uptake and four for the determination of carbon dioxide. Fifteen minutes after the vessels had been placed in the bath at  $37^\circ$ , the gas exchange was determined for a 15 minute period. Growth was then initiated by tipping ammonium chloride from the side arms. Thereafter the respiration was measured at regular intervals and members of each

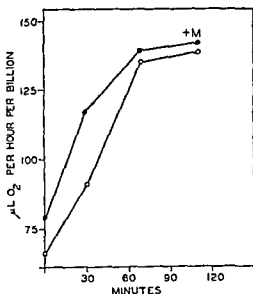


FIG. 6. THE CHANGE IN OXYGEN CONSUMPTION PER CELL IN BUFFER-GLUCOSE SOLUTION WITH AND WITHOUT METHIONINE WHEN GROWTH IS INITIATED BY THE ADDITION OF AMMONIUM CHLORIDE

set were removed from the bath in sequence to determine the amount of growth photometrically. This was checked by viable plate counts on the set removed at 30 minute periods. At the same time, the carbon dioxide evolution was measured in the appropriate set.

Figure 6 illustrates the change in the oxygen consumption per cell ( $RO_2$ ) during the course of the experiment. Before the ammonium chloride was tipped into the well, the  $RO_2$  of the cells incubated with methionine was 79, or 20 per cent more than that of the cells incubated without methionine. This again demonstrated the ability of methionine to preserve the respiration of resting cells. Following the initiation of growth by tipping  $NH_4Cl$ , the  $RO_2$  of the methionine treated cells rose to 142 while that of the control cells increased to 139 during the course of 1.5 hours. This increase is characteristic of the change from resting to actively growing cells and is achieved more



pidly in the presence of methionine. It probably represents the extra energy required for synthesis and multiplication.

Figure 7 illustrates the manner in which the final logarithmic rate of growth (the growth constant) is achieved. The values of  $K$  were calculated from the formula

$$K = \frac{2.3}{T_2 - T_1} \log \frac{N_2}{N_1}$$

using two sets of data: the rates of oxygen uptake and the photometric measurements of the number of bacteria.  $KO_2$ , derived from the oxygen uptake, probably represents the rate of increase in active respiratory enzymes while

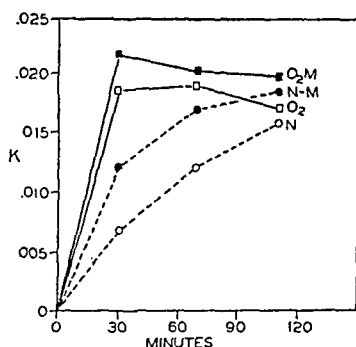


FIG. 7. THE CHANGE IN GROWTH CONSTANTS OF *E. COLI* IN BUFFER GLUCOSE SOLUTION WHEN GROWTH IS INITIATED BY THE ADDITION OF AMMONIUM CHLORIDE

$N$ , the growth constant calculated from photometric number of organisms in plain medium;  $N-M$ , the growth constant calculated from the photometric number of organisms in medium containing methionine;  $O_2$ , the growth constant calculated from rate of oxygen uptake of organisms in plain medium;  $O_2M$ , the growth constant calculated from rate of oxygen uptake of organisms in medium containing methionine.

$N$ , based on the number of bacteria, is the usual growth constant. Curves based on carbon dioxide production are not included since they paralleled the oxygen consumption both in the presence and absence of methionine. The values of the R.Q. at the four points were 103, 110, 106, and 106 consecutively.

The data in figure 7 lead to two conclusions.

(1) The maximum rate of increase in respiratory enzymes is attained at least one hour before the maximum rate of cell division. At the second hour they are approximately equal and constant. Hence the rate of respiration per cell must rise and then become constant as shown in figure 6.

(2) Methionine accelerates the increase in rate of both growth and oxygen consumption (i.e., shortens the induction period), but does not appreciably alter the growth constants once the logarithmic rate of increase is achieved.



In the presence of methionine it takes only half as long as in the control to reach 50 per cent of the maximum growth rate.

Presumably it is one or more of these reactions which is stopped by the sulfonamides and which is reinstated upon the addition of methionine. In view of recent experimental work on transmethylation (3, 4), we have tested for antagonistic activity choline (from  $10^{-7}$  to  $10^{-4}$  M) both alone and in the presence of homocystine ( $10^{-6}$  and  $10^{-4}$  M), but with negative results.

#### DISCUSSION

Evidence has been presented in the body of this paper that at least three different types of antagonist against the sulfonamides can be demonstrated with the aid of *E. coli.*, and that each of these acts in a different way, i.e., circumvents the action of the drug at a different point in the bacterial metabolism. Methionine is active against only the lower concentrations of any sulfonamide, whereas an unknown substance in proteose-peptone is active against some effect only produced by high concentrations of sulfapyridine, sulfathiazole, and sulfadiazine, but not sulfanilamide. *p*-Aminobenzoic acid is active against all concentrations.

The similarity in chemical structure between *p*-aminobenzoic acid and sulfanilamide, and their concentration relationships in antagonism experiments, indicate a competitive inhibition, as suggested by Woods (1). The acid, or some very closely related substance, enters into some reaction within the cell, for example, at an enzyme surface. Sulfanilamide can attach to the surface, but cannot react, and therefore blocks the process. The antagonistic action of methionine, however, must involve another reaction, for the structure of methionine is different from that of sulfanilamide. In addition, methionine antagonizes the inhibitions caused by ethionine, norleucine and norvaline, which *p*-aminobenzoic acid is unable to do. These seem to be competitive inhibitions, and we may suppose that ethionine, for example, endeavors to enter reactions involving methionine, but cannot carry them out. Methionine and *p*-aminobenzoic acid, therefore, must act at different, though related, loci within the bacterium.

In order to relate the data of this paper with the theory presented previously (2), we tentatively make use of the scheme presented in figure 8. The scheme represents a section of the bacterial metabolism which is devoted to a number of reactions necessary for growth and which is sensitive to the sulfonamide drugs. The primary reaction involves *p*-aminobenzoic acid, and is the first in the chain. Its products enter into the *secondary reactions* producing a number of products  $X_1 \dots X_n$ . Each of these is necessary for growth, so that a failure or a slowing in the rate of production of any one will result in a slowing or failure of growth.

It should be emphasized that many essential reactions are probably carried



out without the intervention of *p*-aminobenzoic acid. Likewise, some of the actions of the sulfonamides in bacteria may not involve *p*-aminobenzoic acid-dependent systems, as for example, in the action of cozymase in *Staphylococcus* (5) and of nicotinamide in *Shigella* (6).

By means of this scheme we can systematize the data now available. Sulfonamide competes with *p*-aminobenzoic acid in the primary reactions thereby decreasing the rate of synthesis of the *X* group of compounds. When the stores of *X* fall below a limiting concentration, the rate of growth declines. The addition of sufficient *p*-aminobenzoic acid to the culture medium at any time before dissolution of the cell should immediately restore growth since the primary reactions now have available *p*-aminobenzoic acid and the group *X* is regenerated.

When the primary reactions are slowed by displacement of the aminobenzoic acid, the decline in synthesis is not reflected equally among all of the products. Thus when it is inhibited about 30 to 40 per cent, the main effect is the suppression of the formation of one of the *B* series; i.e. methionine, which in turn leads to a shortage of *X*<sub>1</sub>. Since the remainder of the *X* series is formed at

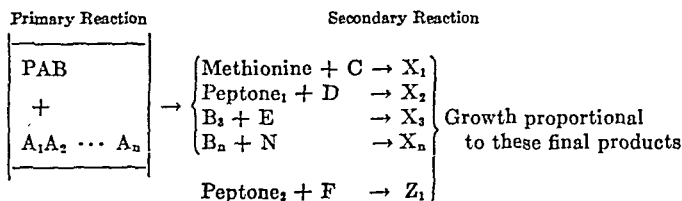


Fig. 8

almost the normal rate, the addition of methionine restores growth. The same type of inhibition, resulting from inadequate *X*<sub>1</sub> can be achieved by the addition of ethionine, norleucine or norvaline which compete with the methionine of the cell just as the sulfonamides compete with *p*-aminobenzoic acid. In an analogous way, the addition of relatively small quantities of methionine restores growth. The fact that *p*-aminobenzoic acid does not relieve the ethionine inhibition places the methionine effect as a reaction secondary to the primary reactions involving *p*-aminobenzoic acid. If methionine were primarily concerned with the formation of *p*-aminobenzoic acid, and overcame the sulfonamide inhibition by increasing the amount of *p*-aminobenzoic acid available to the cells, then the ethionine inhibition of growth caused by displacement of methionine should be circumvented by the addition of *p*-aminobenzoic acid.

As the concentration of sulfanilamide is raised and the primary reactions are increasingly inhibited, the synthesis of the other *X* components, in turn, is inhibited. Thus the inhibition extends first beyond the power of methionine to correct it, then of peptone 1, of peptone 2, and so on. On the other hand,



at least with sulfanilamide, *p*-aminobenzoic acid always remains effective because, by correcting the defect in the primary reaction, it insures the restoration of all the reactions which follow.

The time course of sulfonamide inhibition may also be described by the scheme. It was shown in the previous paper, that for the first hour or so after the addition of sulfanilamide, no inhibition is seen. Apparently the stores of  $X$  have not diminished to the point where they become limiting to cell growth. Later inhibition begins and increases with time. The first inhibition to appear is largely corrected by the addition of methionine and must, therefore, be due chiefly to the failure of  $X_1$ . As contact with larger concentrations of drug continues, other loci are affected and  $X_2 \cdots X_n$  become limiting. Inhibition then occurs in the presence of methionine.

On the basis of the foregoing we propose to label antagonists according to the locus at which they act. *p*-Aminobenzoic acid would be a primary antagonist because it acts in the primary reaction, methionine would be a secondary one.

It should also be noted that the scheme includes a reaction which is not dependent upon *p*-aminobenzoic acid. We suppose that peptone 2 compensates for a derangement of this type which is caused by sulfapyridine, sulfathiazole, and sulfadiazine at high concentrations, but not of sulfanilamide.

Finally, it should be recalled that the various complex culture media will contain varying amounts of primary and secondary antagonists, thus complicating the analysis of drug action. In addition, some bacteria may through evolution have lost synthetic abilities possessed by others, further complicating an interpretation of the results. Methionine, for example, may be required as an essential amino acid by some species, and therefore, its synthesis could not be inhibited (for there would be no synthesis), and it could not serve as an antagonist in these species.

#### SUMMARY

1. Methionine is an antagonist for the action of sulfanilamide, sulfapyridine, sulfadiazine and sulfathiazole on *E. coli*. This action is not due to a non-specific stimulation of growth.

2. Methionine, unlike *p*-aminobenzoic acid, is effective only against low concentrations of the sulfonamides and does not exhibit a simple relationship in the concentrations necessary to antagonize increasing amounts of the sulfonamides.

3. The locus affected by methionine is one of the first to be inhibited by sulfonamide both in the point of time of contact and in the concentration of drug required.

4. Specificity is shown in the chemical and optical configuration. *l*(-) methionine is at least ten times as potent as the isomer and no other related compound carries activity.



5. Ethionine, *norleucine* and *norvaline* inhibit the growth of *E. coli* in simple media. This inhibition is due to competition with methionine.
6. Methionine is neither oxidized, decarboxylated nor deaminated by *E. coli*. It preserves the resting respiration of the cell, decreases the induction period when growth begins, and stimulates the synthesis of active respiratory enzymes.
7. A unified theory is presented to account for the actions of the known antagonists of the sulfonamides. Methionine is an antagonist because the sulfonamides, at low concentrations, act primarily by inhibiting the synthesis of methionine. This synthesis is dependent upon the presence of *p*-amino benzoic acid.

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# RELATIONSHIP OF THE CHEMICAL STRUCTURE OF MORPHINE DERIVATIVES TO THEIR URINARY EXCRETION IN FREE AND BOUND FORMS<sup>1</sup>

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Received for publication August 8, 1941

The discovery of a bound form of morphine in the urine of both animals (1) and man (2) raised the question as to the nature of the substance bound with morphine and on what position or positions of the morphine molecule this binding occurs. The existence of certain compounds of the morphine series in which either one or both the phenolic or the secondary alcoholic hydroxide group of morphine were methylated made it possible to study the position of conjugation. No evidence has been found that a methyl group on either the phenolic hydroxide or the secondary alcoholic hydroxide of morphine is altered or split off by the body.

Since it is well known that phenols and other substances, such as phenolphthalein and pregnandiol, having a phenolic hydroxide, are excreted as glucuronides (3 and 4), it would appear that morphine, which also has a phenolic hydroxide, might also be excreted as a glucuronide. This suggested a study of the concentration of glucuronic acid in urine in patients receiving increasing amounts of morphine.

**METHODS.** The urinary excretion of diacetylmorphine (Heroin), alpha-isomorphine, methyl morphine (Codeine), dihydroheterocodeine, dihydrocodeine, dihydroisocodeine, and dihydrocodeine methyl ether were studied in morphine addicts after substitution (5) for morphine. The urine was analyzed for both the free and bound forms of the alkaloid (2, 6, 7). The colorimetric procedure (6) was used for derivatives of morphine in which the phenolic hydroxide was not methylated. For those compounds in which the phenolic hydroxide was methylated, the silicotungstic acid technique (7) was used. The following slight improvement was made in the latter: the residue from the first liquid-liquid extraction was dissolved in saturated sodium bicarbonate and re-extracted in another liquid-liquid extractor. This gave a much cleaner residue which, after acid and alkaline extractions in a separatory funnel, made it possible to omit the permutit step.

The color intensities or the weights of precipitates in the final residues of all morphine derivatives were compared with known concentrations of the bases of morphine and codeine, respectively. The ratios of concentrations of the bound to the free forms were calculated to indicate extent of conjugation.

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<sup>1</sup> Read before The American Society for Pharmacology and Experimental Therapeutics, Chicago, April 15-19, 1941.



The glucuronic acid of urine was determined by the method of Maughan, Browne, and velyn (8) in patients receiving daily doses of morphine varying from 100 to 4,000 mgm.

**RESULTS.** The average percentage excretions of the various morphine derivatives in both free and bound forms are recorded in table 1. It was found that morphine, alpha-isomorphine, methyl morphine (Codeine), and dihydroheterocodeine are excreted in both free and bound forms. Diacetylmorphine (Heroin) (9) is excreted as morphine in both forms. The bound fraction was 2-10 times greater than the free in every instance, the higher values being associated with larger doses. Dihydrocodeine and dihydroisocodeine were excreted mainly in the free form, only small amounts being bound. Dihydrocodeine methyl ether was excreted only in the free form.

Figure 1 shows the proportionate increase in daily amounts of glucuronic acid excreted in the urine of patients receiving increasing amounts of mor-

TABLE 1  
*Relationship of chemical structure of morphine derivatives to their urinary excretion in free and bound forms*

DRUG	NUMBER OF ANALYSES	DOSAGE RANGE	PERCENTAGE EXCRETION		RATIO OF BOUND TO FREE
			Free	Bound	
		mgm.			
No. 127—Diacetylmorphine.....	30	69-204	5.8	43.5	8.7
No. 112—Dihydroheterocodeine.....	14	63	8.1	37.8	5.5
No. 143—Morphine.....	26	120-600	6.2	32.8	5.4
No. 236— $\alpha$ -Isomorphine.....	16	85-120	4.4	12.0	3.2
No. 2—Codeine.....	23	240-700	11.2	31.6	3.2
No. 51—Dihydroisocodeine.....	19	476-983	18.3	18.4	1.0
No. 397—Dihydrocodeine.....	15	523-440	13.0	8.8	0.73
No. 87—Dihydrocodeine methyl ether....	9	846	22.0	6.6	0.37

phine. The glucuronic acid concentration dropped after a reduction of morphine, and returned to the normal range following withdrawal.

**Discussion.** It would appear that both the phenolic and the secondary alcoholic hydroxide groups of the morphine molecule may be involved in the binding process, for when both of these groups are methylated no conjugation occurs. It is not apparent why this conjugating reaction is diminished in the hydrogenated codeine compounds. Since phenols are excreted largely as glucuronides, and since glucuronic acid is increased in urine with increasing dosages of morphine, it would appear that the substance bound with morphine may be largely glucuronic acid or its lactone form. This union is probably a glucosidic linkage through the aldehyde group to the alkaloidal molecule on either the phenolic hydroxide or the secondary alcoholic hydroxide. This would leave the carboxyl group of the glucuronic acid and the tertiary nitro-



gen of the alkaloid free to behave as a salt in either an alkaline or an acid solution, respectively. Such a substance would be an ampholyte.

Attempts to extract unaltered codeine conjugate with an organic solvent in either an acid or an alkaline solution have been unsuccessful. Since the isoelectric point is not known, the free form of codeine was removed from the urine of a codeine addict and extractions made at pH 5.5, 6.5, and 7.5, respectively. In no instance was codeine found in the receiving flask after boiling the aqueous solution of the residue with acid. When this urine, after the three extractions at the different pH reactions, was then subjected to acid hydrolysis, a strong test for codeine was obtained. Apparently the isoelectric point for the conjugated codeine is not pH 5.5, 6.5 or 7.5.

#### URINARY GLUCURONIC ACID AT VARIOUS MORPHINE DOSAGE LEVELS

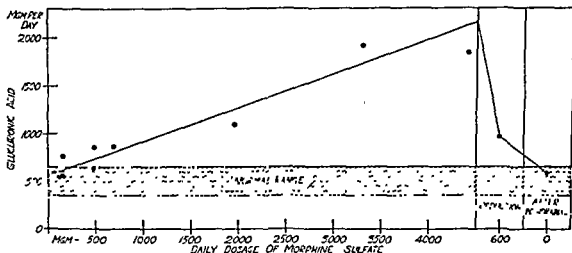


FIG. 1

#### SUMMARY

Morphine, alpha-isomorphine, methyl morphine (Codeine), and dihydroheterocodeine are excreted in both free and bound forms. Diacetylmorphine (Heroin) is excreted as morphine in both forms. Dihydrocodeine and dihydroisocodeine are excreted mainly in the free form, only small amounts being bound. Dihydrocodeine methyl ether is excreted only in the free form. These results indicate that conjugation takes place on either the phenolic or the alcoholic hydroxide.

Since the glucuronic acid concentration in urine increases proportionately with increasing dosages of morphine, the substance bound with morphine and certain of its derivatives is thought to be largely glucuronic acid or its lactone form, glucurone.

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## OBSERVATIONS ON THE TRYPAN BLUE CAPILLARY PERMEABILITY TEST IN RABBITS

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Received for publication August 6, 1941

Various workers have shown that colloidal dyes when injected intravenously normally escape from the circulation relatively slowly and stain all of the tissues fairly uniformly. In areas of inflammation, however, or in areas to which various irritants have been applied, the rate of diffusion is so increased that these parts rapidly become deeply stained, establishing a clear contrast with the faintly colored background. (See Menkin (1) for review and literature.) The local staining at a test site in the skin of the rabbit following the intravenous injection of trypan blue has been employed extensively as a means of demonstrating changes in capillary permeability. In previous experiments (2) we have confirmed the observations of Rocha e Silva and Bier (3) that histamine produces a positive trypan blue reaction and have further shown that various tissue extracts produce positive reactions which can be accounted for by the histamine content of the extracts. Since the test tissue, i.e., the rabbit's skin, contains a considerable amount of histamine, it seemed highly probable that agents which do not contain histamine but which have the property of liberating it from living tissues would on this account produce positive reactions when tested by this method. Ramsdell's (4) observations on the production of trypan blue reactions in sensitized rabbits after the intracutaneous injection of antigen support such a conception. To test this theory further we have studied a number of biological agents which have been shown by various methods to have the property of liberating histamine, some which are known to lack this property, and some for other reasons referred to later.

The biological agents, in addition to antigen in sensitized animals, which have been demonstrated to have the property of liberating histamine from living tissues are certain snake venoms (5, 6) bee venom (7), staphylococcal toxin (8), proteoses (9, 10) and trypsin (11, 12). All of these agents were employed. Since trypsin is a proteolytic enzyme, other proteolytic enzymes were included, particularly in view of the fact that preliminary experiments in our laboratory indicated that pepsin did not have the property of liberating

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TABLE 1

*Trypan blue reaction in rabbits after the intracutaneous injection of various biological preparations*

AGENT*	CONCENTRATION	NUMBER OF EXPERIMENTS	REACTION†
	<i>mgm. per cc.</i>		
Histamine.....	0.001	4	0, 0, +, +
	0.003	4	0, +, +, +
	0.01-0.06	11	All +
	0.1-10.0	10	All ++
Trypsin.....	0.005	1	0
	0.05	1	+
	0.1	2	+, +
	0.5	1	+
	1.0	6	+, +, +++, +++, +++, +
	2.0	6	All +++++

\* Source and description of agents employed:

Histamine—as histamine biphosphate.

Trypsin, chymotrypsin, and pepsin—crystalline preparations from Plaut Research Laboratories. (The concentrations indicated in the table include the accompanying magnesium sulfate.)

Ficin—from Merck, courtesy of Dr. R. T. Major.

Papain—a fairly purified preparation, kindly supplied by Dr. M. Bergmann, Rockefeller Institute. (The preparation was dialyzed prior to use; the concentrations indicated in the table represent weighings prior to dialysis.)

Bacto-Protone and Bacto-Peptide—Digestive Ferments Company (these preparations were dissolved in saline, acidified, shaken with permutit, filtered, and neutralized to remove traces of histamine).

Snake venoms—as dried venom from the Antivenine Institute of America, courtesy of Mr. R. H. Hutchison.

Bee venom—a lyophilized preparation from Sharp and Dohme, courtesy of Dr. J. L. McCartney.

Secretin—a highly purified preparation, courtesy of Dr. Harry Greengard, Northwestern University.

Staph. Toxin—Staphylococcus Aureus Toxin, prepared according to the method of Dolman, and kindly supplied by Dr. G. F. Youmans, Northwestern University.

Insulin—powdered insulin, assaying 20 units per mgm., from Lilly, courtesy of Dr. John H. Waldo.

Heparin—Connaught Laboratories.

Spreading Factor—a highly purified testis hyaluronidase, kindly supplied by Dr. K. Meyer, Columbia University. (Preparation dialyzed prior to use.)

Urogastrone—a highly purified preparation, courtesy of Dr. John Gray, Northwestern University.

Acetyl-choline—acetyl-choline chloride with added physostigmine.

† Character of reaction: 0, negative; +? = doubtful; +, homogeneous blue spot; ++, blue halo around a pale center; +++, blue halo, center initially pale, later showing hyperemia to hemorrhage; +++++, blue halo, center initially pale, later showing hyperemia, hemorrhage, to local digestion or necrosis.



TABLE 1—Continued

AGENT*	CONCENTRATION	NUMBER OF EXPERI- MENTS	REACTION†
	<i>mgm. per cc.</i>		
Chymotrypsin .....	0.005	1	0
	0.05	1	0
	0.1	1	+
	2.0	1	+++
	5.0	1	++++
Pepsin ...	0.1	1	0
	1.0	1	0
	5.0	1	0
	10.0	2	0, +?
	20.0	1	+
Ficin . . . . .	0.01	1	0
	0.05	1	0
	0.1	1	+
	0.5	1	+
	1.0	1	++++
	10.0	1	++++
Papain .. . . .	10.0	1	+
	100.0	2	++++, ++++
Bacto-protone ....	1.0	1	0
	10.0	2	0, +?
	100.0	3	+, +, +
<i>Crotalus terrificus</i> venom‡	0.02	1	+
	0.2	1	+
	0.4	1	+++
	0.5	1	++++
	1.0	1	++++
Bee venom .	1 sting per cc.	1	0
	10 stings per cc.	1	++
Staph. toxin.		2	+, +
Secretin ...	0.1	1	0
	1.0	1	0
	3.0	1	0
Bacto-peptone ....	1.0	1	0
	10.0	1	0
	100.0	1	0

‡ Similar results with venoms of *Crotalus atrox*, *Bothrops atrox*, and *Agkistrodon pisc.*



TABLE 1—*Concluded*

AGENT*	CONCENTRATION	NUMBER OF EXPERIMENTS	REACTION†
	<i>mgm. per cc.</i>		
Insulin.....	0.5	1	0
	1.0	2	0, 0
	10.0	2	0, +?
	100.0	1	0
Heparin.....	0.1	1	0
	0.5	2	0, 0
	10.0	1	0
Spreading-factor.....	0.5	1	0
	1.0	1	0
	2.0	2	0, 0
Acetyl-choline.....	0.01	2	0, 0
	0.1	1	0
	1.0	1	0
	10.0	2	0, +?
Urogastrone.....	1.0	2	0, +?
	2.5	1	+

histamine. Bacto-protone was employed as a convenient proteose preparation and Bacto-peptone was used as a control agent since it is comparable to Bacto-protone except that it contains relatively little proteose nitrogen and previous experiments (13) have shown that it does not produce "peptone shock" as does Bacto-protone. The "spreading factor" of Duran-Reynals was studied since Rigdon (14) had found that testis extracts containing this factor produce a positive trypan blue reaction, while Menkin (1) has reported that the factor can be dissociated from the capillary permeability factor of testis extracts. Insulin and heparin were employed since observations in our laboratory indicated that these preparations do not have the property of liberating histamine. Secretin and urogastrone were included as representatives of untested biological preparations and acetyl-choline was used in order to compare its effects with those of histamine, since it may be considered as a highly active tissue constituent that might be liberated under the same conditions which result in the liberation of histamine.

#### EXPERIMENTAL

The tested substances were injected intracutaneously in the abdominal area of rabbits in an amount of 0.3 cc. The rabbits were shaved 24 hours prior to the experiment except in some instances in which the fur was clipped closely on the same day. Since preliminary observations indicated that young,



immature animals give variable results, only adult white rabbits weighing 3 to 4 kilograms were employed. Immediately after the intradermal injections, 10 cc. of 1 per cent trypan blue was injected *via* a marginal ear vein, and the reaction at the test site closely followed. Previous experiments had demonstrated that the reactions with histamine varied according to the concentration of the solution employed. Concentrations less than 0.1 mgm. per cubic centimeter produce a homogeneous blue spot, while concentrations greater than this produce a blue halo around a central pale area. Accordingly the tested substances were employed in varied concentrations. The results are shown in the accompanying table.

#### DISCUSSION

As noted in the table, all of the agents (snake venoms, bee venom, staphylococcus toxin, proteose, trypsin) known to have the property of liberating histamine from living tissues produce a positive trypan blue reaction, while the agents (heparin, insulin) known to lack this property yield negative tests. It would appear that this test might be employed as provisional evidence for the ability of a given agent to liberate histamine. That it is not infallible in this regard was shown by one of us (15) in some experiments with "Kallikrein," and is indicated furthermore by the improbability that histamine is the only substance capable of increasing capillary permeability. On the other hand, a negative trypan blue test would appear to be highly significant as excluding histamine, either as a contaminant of the preparation employed or as an intermediary reactant liberated by the preparation. As a corollary to these observations it may be noted that the trypan blue test has been employed as evidence to indicate that a particular agent, operating *per se*, has the property of increasing capillary permeability. The results here reported indicate that such a generalization is unwarranted since the particular agent may act only indirectly by liberating histamine.

In the effort to increase the definitive value of a positive trypan blue test as evidence for the liberation of histamine, a number of experiments were performed with arginin. Ackermann (16) demonstrated that arginin and related substances exhibit a specific antagonism to the effects of histamine on the guinea pig intestine. We have confirmed this finding, but we were unable to confirm his additional report that arginin specifically antagonizes the vascular effects of histamine in dogs. Similarly, arginin did not prevent the production of a trypan blue reaction by histamine or by any of the agents which liberate it. Arginin alone, in adequate concentrations (10 mgm. per cubic centimeter) was able to produce a positive reaction.

As indicated in the table, a number of agents which produced a simple positive trypan blue reaction in weak concentrations, produced additional changes in stronger concentrations. While the complete effect (homogeneous blue spot) produced by weak concentrations, and the blue halo produced by



strong concentrations, can be accounted for by the local release of histamine, the superimposed hyperemia, hemorrhage and necrosis must be attributed to a specific effect of the agents employed since histamine alone is unable to produce these effects. This interpretation is supported by the observation that the effects of protease, which does not have the necrotizing effect of the snake venoms, etc., does not extend beyond that produced by histamine.

The "spreading factor" of Duran-Reynals did not produce a positive reaction. This is in agreement with the observations of Meyer (17) and with the statement of Menkin that the capillary permeability factor and spreading factor of testis extracts are not identical and would indicate that the positive reactions which Rigdon obtained with crude testis extracts are probably similar to those which can be obtained with any tissue extract containing traces of histamine. When spreading factor was injected with histamine, it acted to increase the size of the discolored area and to decrease the intensity of the staining, indicating that it did not increase the amount of dye diffusing through the capillaries but acted to dilute its local concentration by aiding its diffusion through the adjacent tissues. Further evidence for this conclusion was presented by the observation that a dose and concentration of a proteolytic enzyme which, when injected alone, produced considerable local necrosis, failed to do so when "spreading factor" was combined with it while the area of hyperemia was considerably increased. In contrast to this effect of "spreading factor," histamine, when injected in combination with a proteolytic enzyme, increased the amount of local necrosis. If alterations in capillary permeability are due to changes in the intercellular cement substance of the capillary endothelium (18), while the spreading properties of hyaluronidase are due to changes in the intercellular cement substance of the tissue cells, the conspicuously contrasting effects of histamine and of spreading factor would indicate a corresponding difference in the nature of the cement substance in the respective areas.

The negative results with acetyl-choline indicate that this substance, a cellular constituent that might be liberated in a fashion similar to histamine, could not be concerned in the increased capillary permeability which various biological preparations produce.

#### CONCLUSIONS

1. A number of biological substances (snake venoms, bee venom, staphylococcal toxin, protease, proteolytic enzymes) which have the property of liberating histamine from living tissue, produce positive trypan blue reactions in rabbits, and no substance known to have this property has been found to give a negative test.

2. The trypan blue test in rabbits is a simple procedure for the provisional identification of the histamine-liberating capacity of a preparation in that a positive reaction is suggestive evidence for such a property, while a negative reaction is significant evidence for the absence of such a property.



3. The production of a trypan blue reaction in rabbits by a particular agent cannot be considered as evidence that the particular agent acts *per se* to increase capillary permeability, since it may do so indirectly by liberating histamine.

4. Arginin, although it is a specific antagonist against some of the effects of histamine, does not prevent the production of a trypan blue reaction by either histamine, or agents which liberate histamine.

5. The "spreading factor" of Duran-Reynals (testis hyaluronidase) does not produce a trypan blue reaction, but has a "spreading effect" upon reactions that have been produced by other agents.

6. Acetyl-choline does not produce a positive trypan blue reaction, and cannot be considered, therefore, as an intermediary substance comparable to histamine for the production of an increased capillary permeability.

We gratefully acknowledge the assistance of Franz Gotzl and H. L. Terry in some of the experiments.

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# THE USE OF CLORARSEN IN THE TREATMENT OF SYPHILIS<sup>1,2</sup>

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Received for publication August 9, 1941

Arsenoxide was first studied by Ehrlich and Hata (1) but discarded because it was thought to be too toxic. It has recently been reintroduced through studies by Tatum and Cooper (2) in animals and by Foerster and his co-workers (3) in the treatment of human syphilis. Since its introduction it has received wide clinical use and its value has become generally accepted. Because of the advantages which arsenoxide seems to possess in effectiveness in the treatment of syphilis, ease of administration and low toxicity, it was felt desirable to investigate the properties of another drug of similar nature. The drug 3-amino-4-hydroxy-phenyl dichlorarsine hydrochloride, now known as Clorarsen, has been subjected to experimental and clinical trial. The purpose of this paper is to present the results of this study.

3-amino-4-hydroxy-phenyl dichlorarsine hydrochloride was studied in animals by Ewins and Everett (4) and Hall (5). It was found to be effective in yaws by Chesterman and Todd (6, 7, 8) and later was tried in five cases of human syphilis by Levaditi, Pinaud and Even (9) who found it to be unsatisfactory. These studies were apparently all made using an unbuffered aqueous solution. Such a solution would have a pH below 1.5 which we consider undesirably low and may account for Levaditi's unsatisfactory results and for the lack of further studies of the drug.

Clorarsen is a mixture of 3-amino-4-hydroxy-phenyl dichlorarsine hydrochloride and a buffer salt, sodium citrate. It is supplied in ampoules in dry powder form and is readily soluble in the required quantity of distilled water, about 10 cc. per dose to form a clear, colorless solution having a pH of about 5.2. The drug is very stable and has been kept in powder form in ampoules at ordinary temperatures (22° to 30°C.) for 23 months without evidence of any change. It has similarly been kept at 50°C. for 94 days without alteration in composition.

It is generally believed that on neutralizing the above arsenical the corresponding oxide is found, i.e., 3-amino-4-hydroxy-phenyl arsine oxide, commonly known as arsenoxide, to which has been ascribed the therapeutic activity of arsphenamine. Presum-

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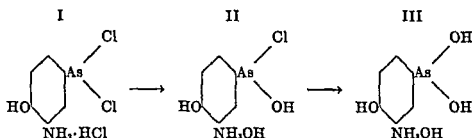
<sup>1</sup> Aided by Grants from Hopkinson Laboratories, E. R. Squibb and Sons, and the Barbara Henry Research Fund.

<sup>2</sup> The authors wish to acknowledge their indebtedness to the resident staff and the staff of the syphilis clinic of the Department of Medicine of the New York Hospital and Cornell University Medical College for their cooperation throughout this experiment.



ably, therefore, the spirocheticidal effect of clorarsen is due to the formation of arsenoxide or more likely its hydrated form in the blood stream.

As a solution of 3-amino-4-hydroxy-phenyl dichlorarsine hydrochloride is raised toward neutrality the chlorine groups probably are successively replaced by hydroxyl groups, thus:



When the mixture which constitutes clorarsen is dissolved in water the arsenical in all probability is converted to compound II and part of it to compound III. In addition, the use of sodium citrate in clorarsen has a favorable effect on toxicity which is not clearly understood. As will be seen in table 2, 23 out of 25 rabbits survived intravenous injections of 15 mgm. per kgm. of clorarsen. When the sodium citrate was replaced by

TABLE 1  
*Toxicity of clorarsen in mice*

NUMBER OF ANIMALS	DOSE	SURVIVED	DIED
	<i>mgm. per kgm.</i>		
31	50.5	12	19
22	45.0	10	12
42	39.2	34	8
10	33.6	10	0

sufficient sodium carbonate to produce the same pH only 8 out of 20 rabbits survived the same dosage.

*Toxicity in mice.* A series of mice was injected intraperitoneally with varying doses of Clorarsen and observed for a period of 2 weeks. The results are recorded in table 1. The mean toxic dose (L.D. 50) is seen to be 45 mgm. per kilogram.

*Toxicity in rabbits.* A number of rabbits was injected with varying quantities of Clorarsen and observed for at least two weeks. The results are recorded in table 2. These figures indicate that the L.D. 50 is approximately 17 mgm. per kilogram.

Further studies were done to determine the effect of repeated injections of Clorarsen in rabbits over a long time period. A series of animals were given varying doses of Clorarsen over a period of 4 weeks as indicated in table 3. All the animals treated survived except one not included in the above table. This animal died of intercurrent infection during the first week of the experiment. After the conclusion of the injections the animals were sacrificed and examined. No gross lesions were noted. Sections of the livers and kidneys



were examined microscopically by the resident pathologist whose opinion was that they showed no changes which could be interpreted as due to the toxic effect of the drug.

*Clinical study.* The material for this study consists of a total of 171 patients treated over the 2-year period from August 1939 to August 1941. Of these, 117 were early cases, 48 of whom have been treated for more than 6 months, and 40 for more than one year. The distribution of cases is shown in table 4. There were 9 with seronegative primary syphilis, 25 with seropositive primary syphilis, 64 with secondary syphilis, 17 with both primary

TABLE 2  
*Toxicity of clorarsen in rabbits after single injections*

NUMBER OF ANIMALS	DOSE	SURVIVED	DIED
	<i>mgm. per kgm.</i>		
21	18	8	13
11	17	5	6
25	15	23	2

TABLE 3  
*Toxicity of clorarsen in rabbits after multiple injections over a four week period*

RABBIT NUMBER	INDIVIDUAL DOSE	NUMBER OF INJECTIONS	TOTAL DOSAGE
	<i>mgm. per kgm.</i>		<i>mgm.</i>
1	1.0	8	20.4
2	1.0	8	16.2
3	1.0	8	19.4
4	1.0	8	16.5
5	2.2	8	21.3
6	2.2	8	21.3
7	2.2	8	21.3
8	2.2	8	21.3
9	2.2	8	23.5
10	3.0	12	72.0
11	5.0	12	120.0

and secondary lesions and 2 early latent cases. There were 42 late cases, all late latent syphilis except four who had asymptomatic neurosyphilis. Of the late cases 30 have been treated for more than 6 months, 26 for more than one year. In addition, temporary therapy was given to 12 patients with various forms of syphilis, in most cases individuals who had had reactions to other drugs. A total of 2,773 injections has been given in the following dosages:

GRAMS	INJECTIONS	GRAMS	INJECTIONS
0.01	21	0.045	1188
0.02	23	0.067	1508
0.03	33		



The general scheme of treatment followed was that of the usual combined bismuth and arsenical treatment given in alternate courses as recommended by Moore (10). The courses of Clorarsen, however, were generally prolonged to 10 or 12 injections. In a few cases, Clorarsen was given bi-weekly for part of the time in courses of 6 to 8 weeks. When given bi-weekly the dosage was 0.045 gram for each injection. When given weekly the dosage was 0.067 gram regardless of sex, except in those instances where reactions were encountered in the larger dosage and not in the smaller. The patients with early syphilis were in almost every instance hospitalized for a period of 7 to 10 days.

*Rate of disappearance of organisms and healing of lesions.* Fourteen early cases of darkfield positive primary or secondary syphilis were followed with darkfield examinations to determine the rate of disappearance of the surface organisms. The patients were given one injection of Clorarsen 0.045 gram in the morning and a darkfield examination of the originally darkfield positive lesion and other lesions was done between 12 and 48 hours after the injection.

TABLE 4  
*Distribution of cases*

<b>Early</b>	
Seronegative primary syphilis.....	9
Seropositive primary syphilis .....	25
Secondary syphilis .....	64
Primary and secondary syphilis .....	17
Early latent syphilis .....	2
<b>Late</b>	
Late latent syphilis .....	38
Asymptomatic neurosyphilis ....	4

In every instance except one, the darkfield was negative when repeated. The actual times of examination in the various cases were as follows: 2 at 12 hours, 4 at 24 hours, 4 at 36 hours, 4 at 48 hours. In one case the darkfield was still positive 12 hours after the first injection but was negative when repeated 36 hours later.

Healing of the primary and secondary lesions was prompt in every case, and compares quite favorably with that obtained by the use of the standard arsenical drugs. No actual measurement in terms of days was made as it is felt that the time for complete healing depends on the size and extent of the individual lesions. We feel, however, that the progress of healing of the individual lesions was excellent.

*Serologic response.* The serologic response to Clorarsen therapy was followed by the Wassermann reaction of samples taken at the inception of therapy and at the end of each course. The results are recorded in table 5. Of the early cases, 40 have had complete reversal of serology within less than



6 months after beginning therapy. Seven more early cases have had delayed reversal of serology, 3 after 7 months of therapy, 1 after 8 months, 2 after 9 months and one after 12 months. Of 48 early cases, originally seropositive, treated more than 6 months, only 3 have failed to show serological reversal. The exact data on these three cases are of interest. The first, a patient with secondary syphilis, has received in one year 34 doses of Clorarsen. The Wassermann was originally positive in 1:2 dilution and now gives a doubtful reaction undiluted. The second patient had a darkfield positive chancre of the lip. He has received 32 doses of Clorarsen in a year. The Wassermann reaction, originally positive in 1:4 dilution, is still positive in 1:2 dilution. Physical examination and spinal fluid are negative. The third patient had secondary syphilis with a Wassermann reaction positive in a dilution of 1:32. This patient received 19 doses of Clorarsen in a period of 7 months and then lapsed treatment. The Wassermann reaction was still positive in 1:8 dilution at the time of lapse. In summary, of 50 early cases, 40 had serologic reversal

TABLE 5  
*Serologic reversals*

<b>Early syphilis</b>	
Serology reversed in less than 6 months.....	40
Serology reversed between 6 and 12 months.....	7
Serology failed to be reversed after 6 months treatment.....	3
<b>Late syphilis</b>	
Serology reversed in less than 1 year.....	10
Serology reversed after 1 year.....	1
Serology failed to be reversed after 1 year treatment.....	10

in less than 6 months, 7 in 6 to 12 months and 3 are still seropositive after more than 6 months therapy.

Of the late cases, who have received more or less continuous therapy, 10 have had serologic reversal within 12 months. One serologic reversal occurred after 19 months of treatment. There were 10 cases of late latent syphilis considered to be seroresistant in that they have received more than one year of treatment without reversal of the serology. In 7 of these there has been definite decrease in titre of reagin.

*Spinal fluids.* Lumbar punctures have been done in 34 cases of early syphilis who have received more than 6 months of therapy. Most of the spinal fluid examinations were made between 6 and 12 months after institution of therapy. Three were first examined more than a year after beginning treatment. Of the 34 cases, 32 had negative spinal fluids. One had a definite Group II fluid 9 months after beginning treatment. The patient had started treatment in the secondary stage and had received 30 injections of Clorarsen in 9 months. The blood serology had become negative in 3 months. When



the positive spinal fluid was found Clorarsen was continued and the fluid was negative 8 months later. The other patient had a slight abnormality of the spinal fluid which was found to be a laboratory error. The lumbar puncture was immediately repeated and found to be negative.

Therefore, of 34 cases of early syphilis treated more than 6 months, 33 had negative spinal fluids.

*Relapses.* Only one case of infectious relapse was seen. This patient, a 22 year old colored girl, was started on treatment in the secondary stage. She received 10 doses of 0.045 gram of Clorarsen in 8 weeks, then lapsed for 5 months, returning at the end of this time with a cutaneous relapse. These lesions cleared after 2 further injections and she again lapsed for 7 months. She again returned with a mucocutaneous relapse.

TABLE 6

*Reactions encountered in 2773 injections of clorarsen in 171 patients*

TYPE OF REACTION	NUMBER OF PATIENTS
Gastrointestinal	
a) Single reaction	27
b) Repeated reactions	5
Syncope	4
Skin Reactions	
a) Exfoliative dermatitis	1
b) Fixed arsenical eruption	1
c) Papular dermatitis	2
d) Pruritis	1
e) Angioneurotic edema	1
f) Ninth-day erythema	2
g) Questionable arsenical dermatitis	1
Chills, fever, headache	2
Jaundice	1
Generalized pains in body	1
Loin pain	1
Questionable purpura	1

No case of serologic relapse was encountered.

*Treatment reactions.* Table 6 shows the character and number of reactions to Clorarsen in the total of 2,773 injections. Various reactions occurred in 51 of the 171 patients treated, seven patients having more than one type of reaction. It is noteworthy that no severe immediate reactions occurred. There were no deaths.

*Gastrointestinal.* As is usual in the use of arsenical drugs the gastrointestinal reactions, nausea, vomiting, diarrhea, were most commonly seen. They occurred on single occasions in 27 patients and repeatedly in 5 patients. Rarely was it necessary to discontinue the drug. In most patients it was found that doses of 0.045 gm. were well tolerated after gastrointestinal reac-



ns to 0.067 gm. The disagreeable taste so frequently accompanying injections of arsenicals was notably absent.

*Syncope.* Fainting shortly after injection of Clorarsen was seen 4 times. In all cases the drug was well tolerated later. These were not nitritoid reactions.

*Skin reactions.* A variety of skin reactions was seen. One case of moderately severe exfoliative dermatitis occurred. There was one case of a fixed arsenical dermatitis. The patient was subsequently able to take full dosage of arsphenamine without difficulty. Two instances of a mild papular pruritic eruption occurred, and further arsenicals were thought to be contraindicated. One case of a dermatitis of uncertain nature occurred. This patient also has later been able to take other arsenicals without further difficulty. Generalized pruritus occurred in one patient after his second dose of Clorarsen. He has subsequently taken full doses of the drug without return of this symptom. Two cases of ninth-day erythema were seen.

*Miscellaneous.* The various miscellaneous reactions are listed in table 6. These include one case of jaundice, two patients who experienced chills, fever, and headache after Clorarsen, one with generalized pains in the body. One patient had loin pain after the first injection without other symptoms and without return of these symptoms after subsequent injections. One patient developed several purpuric spots in the skin while under Clorarsen therapy. He did not return for blood studies and the nature of the purpura was not determined.

### CONCLUSIONS

A new drug, Clorarsen, (3-amino-4-hydroxy-phenyl dichlorarsine hydrochloride, buffered with sodium citrate) has been studied as to its toxicity in animals and its therapeutic value in the treatment of syphilis.

Data are presented on a series of animal experiments and on a clinical study of 171 patients treated during the past two years.

It is felt that Clorarsen is a safe and effective drug in the treatment of syphilis as judged by: 1) rapid production of darkfield negativity of early lesions, 2) prompt healing of early lesions, 3) effectiveness in producing seronegativity in early syphilis, 4) low percentage of relapses, 5) low incidence of abnormal spinal fluids in early syphilis, 6) absence of severe immediate reactions to its administration and relatively low number of reactions in general.

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## A COMPARISON OF CALCIUM ACETYLSALICYLATE (AS CALSAMATE) TO ACETYLSALICYLIC ACID<sup>1</sup>

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Received for publication August 11, 1941

After initial studies on the analgesic and antipyretic properties of acetylsalicylic acid, many attempts were made to enhance its physiological and therapeutic effects. It was found that the water soluble salts of acetylsalicylic acid were better tolerated than acetylsalicylic acid as such since systemic disturbances were reported to occur less frequently following their administration (1 to 8). This is especially true for gastric irritation. Of the various salts investigated, calcium acetylsalicylate was found to be the most satisfactory. It proved to be unstable, however, and combinations of acetylsalicylic acid with calcium compounds which would produce calcium acetylsalicylate in water were generally unstable, insoluble, or hygroscopic.

Calsamate is composed of 60.6 per cent acetylsalicylic acid and 39.4 percent calcium glutamate. The amount of calcium glutamate is slightly in excess of the amount chemically equivalent to the acetylsalicylic acid. When calsamate is placed in water, the acetylsalicylic acid and calcium glutamate react to form calcium acetylsalicylate and glutamic acid.

This study is a comparison of calsamate and acetylsalicylic acid with respect to excretion in humans; gastric irritating properties in rats, rabbits, and dogs; effects on urea clearances and plasma carbon dioxide combining power in dogs; chronic toxicity studies in rats; and acute toxicity studies in rats and rabbits. *In every experiment the doses of acetylsalicylic acid and calsamate were equivalent in acetylsalicylic acid content.*

*Excretion studies in man.* Two series of urinary excretion studies were performed on 14 young adult males with no history of gastric pathology. Acetylsalicylic acid was administered as a *suspension* in doses of 600 mgm. in 250 cc. of water, and the equivalent amount of calsamate was given as a solution in the same amount of water. In the first series the drugs were administered 14 hours after meals, in the second 2 hours after meals. Each subject received each drug.

The bladder was emptied at the beginning of the test. Urine collections

<sup>1</sup> Aided in part by grants from the Wisconsin Alumni Research Foundation and the Lakeside Laboratories, Inc., Milwaukee, Wisconsin.



were then made at 15 minute intervals during the first hour and at convenient intervals thereafter. Collections were continued until the urine was negative or only slightly positive with respect to salicylic acid. Eighteen to 24 hours usually were required. Aliquot portions of each sample were analyzed for salicylic acid by a modification of the method of Hanzlik and Presho (9). Figure 1 is a composite of the 14 individual curves of excretion when the two salicylates were administered post absorptively. Figure 2 contains similar data for 12 subjects who received the medication 2 hours after lunch. The rate of excretion of salicylic acid following administration of calsamate or acetylsalicylic acid is essentially the same. This is interpreted as indicating that the rate of absorption is also the same. Excretion of each was practically

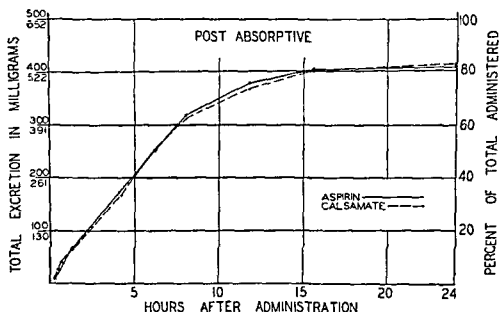


FIG. 1. A COMPOSITE OF 14 INDIVIDUAL CURVES OF EXCRETION FOLLOWING THE ADMINISTRATION OF A 10 GRAIN DOSE OF CALSAMATE AND AN EQUIVALENT DOSE OF ACETYSALICYLIC ACID TO ADULT HUMANS

Values above the line are salicylic acid, those below are the calculated acetylsalicylic acid expressed in milligrams

complete 16 hours after administration, and in each case approximately 80 per cent was recovered. The rate of excretion does not differ significantly when the drugs are given early or late after meals.

*Studies on gastric irritation.* In order to compare the gastric irritating properties of calsamate and acetylsalicylic acid experiments were carried out on a total of 29 rats, 75 rabbits, and 61 dogs. Adult rats were given a daily dose of 300 mgm. acetylsalicylic acid per kilogram or the equivalent amount of calsamate by stomach tube in 5 cc. of water. The animals were sacrificed on the eleventh day and the stomachs removed and opened along the lesser curvature. The stomach contents were washed out with a stream of water and the mucosa observed for signs of irritation.



The results are summarized in table 1. The degree of ulceration in this and succeeding experiments was classified on the following basis:

Several pin point ulcers.....	mild
Several pin point ulcers or a few deep ulcers 3 mm. in diameter....	medium
Several deep ulcers 3 mm. or larger in diameter, or large superficial eroded areas.....	severe

Such a classification is admittedly arbitrary, but since experiments with both drugs were done simultaneously, the method was considered satisfactory. Nine of the 10 rats receiving acetylsalicylic acid showed ulceration, while 4 of 9 given an equivalent amount of calsamate showed some degree of injury

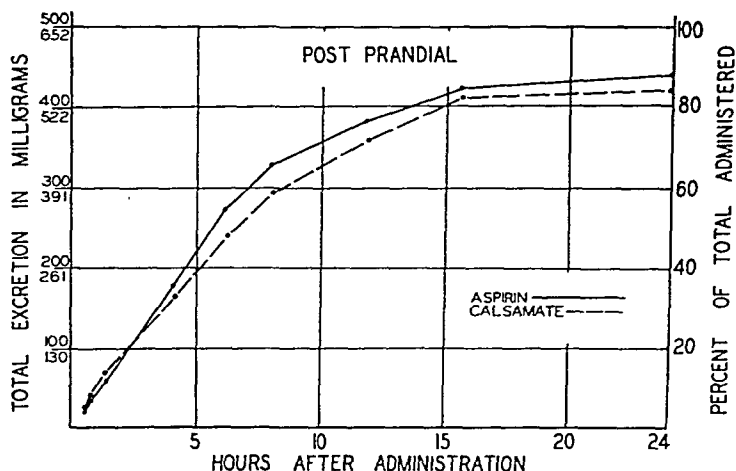


FIG. 2. A COMPOSITE OF 12 INDIVIDUAL CURVES OF EXCRETION FOLLOWING THE ADMINISTRATION OF A 10 GRAIN DOSE OF CALSAMATE AND AN EQUIVALENT DOSE OF ACETYSALICYLIC ACID TO HUMANS

Values above the line are salicylic acid; those below are the calculated acetylsalicylic acid expressed in milligrams

to the gastric mucosa. No ulcers were found in the stomachs of 10 non-medicated animals which served as controls.

The effects of single large doses of calsamate or acetylsalicylic acid were observed on the stomachs of healthy adult rabbits which had not been fed for 24 hours prior to medication. The drugs were given by stomach tube in 100 cc. of cool water. Animals receiving doses of 600 mgm. per kilogram or less were killed  $3\frac{1}{2}$  to 4 hours after administering the drug, and those receiving higher doses were killed 24 hours later. The data from these experiments also are in table 1. All 10 of the animals receiving 300 mgm. of acetylsalicylic acid per kilogram had gastric ulcers. Of an equal number with calsamate only half showed ulceration, and the injury was of a lesser degree of severity.



With doses of 600 mgm. per kilogram the difference in severity of damage produced by the two salicylates was not so definite as with the lower doses. No difference in degree of reaction was distinguishable with doses of 800 and 1000 mgm. per kilogram. Figures 3a and 3b are representative photographs of stomachs of animals treated with 500 mgm. acetylsalicylic acid per kilogram as aspirin and calsamate respectively.

TABLE 1

*Comparison of gastric irritating properties of acetylsalicylic acid and calsamate (as the equivalent amount of acetylsalicylic acid) in rats and rabbits*

NUMBER OF ANIMALS	DRUG	DOSEAGE	DEGREE OF ULCERATION (AS INDICATED IN TEXT)
Rats—10 daily doses			
10	Acetylsalicylic acid	300	1 normal; 4 medium; 5 severe
9	Calsamate	300	5 normal, 1 mild; 2 medium; 1 severe
10	None	Controls	10 normal;
Rabbits—single dose			
5	Acetylsalicylic acid	150	4 mild, 1 medium
5	Calsamate	150	2 normal, 3 mild
10	Acetylsalicylic acid	300	2 medium; 8 severe
10	Calsamate	300	5 normal; 2 mild; 1 medium; 2 severe
2	Acetylsalicylic acid	500	2 severe
2	Calsamate	500	1 mild, 1 medium
2	Acetylsalicylic acid	600	2 severe
2	Calsamate	600	2 medium
7	Acetylsalicylic acid	800	2 medium, 5 severe
8	Calsamate	800	3 mild, 5 severe
6	Acetylsalicylic acid	1000	6 severe
8	Calsamate	1000	1 mild; 7 severe
8	None	Controls	6 normal; 2 mild

A study of the gastric irritating properties of calsamate and acetylsalicylic acid in dogs showed that daily doses of 25 and 50 mgm. per kilogram of either substance for 7 days produced no gastric ulcers. Administration of 100 mgm. acetylsalicylic acid per kilogram twice daily for 5 days caused ulceration in all 3 animals, while the equivalent dose of calsamate to 3 other animals produced no ulcers. When this dose of calsamate was given for 11 days, there





FIG. 3a. PHOTOGRAPH OF THE CARDIAC PORTION OF THE STOMACH OF A RABBIT WHICH HAD BEEN GIVEN ACETYLSALICYLIC ACID, 500 MGM. PER KILOGRAM  
The condition illustrated is typical of a "severe" degree of ulceration. Approximately  $\frac{2}{3}$  natural size



FIG. 3b. PHOTOGRAPH OF THE CARDIAC PORTION OF STOMACH OF A RABBIT WHICH HAD BEEN GIVEN ACETYLSALICYLIC ACID, 500 MGM. PER KILOGRAM AS CALSAMATE  
With the exception of a superficially eroded area the stomach was normal. Approximately  $\frac{2}{3}$  natural size



were a few pin point ulcers. The results are listed in table 2. Figures 4a and 4b are photographs of stomachs from an animal in each group. Ten non-medicated controls had no gross evidence of gastric pathology.

Five additional dogs were given a daily dose of 50 mgm. of acetylsalicylic acid per kilogram and 4 an equivalent amount of calsamate for 23 days. Food was removed from the cages approximately 15 hours and water 2 hours before administration of the drugs, which were given by stomach tube in a volume of water equivalent to 2 cc. per kilogram. Two hours after the last

TABLE 2

*Comparison of gastric irritating properties of acetylsalicylic acid and calsamate (as the equivalent amount of acetylsalicylic acid) in dogs*

NUMBER OF ANIMALS	DRUG	DOSAGE	DAYS ADMINIS-TERED	DEGREE OF ULCERATION AS INDICATED IN TEXT				
Gross pathology								
		mgm. per kgm. per dose	times per day					
2	Acetylsalicylic acid	25	7 s.i.d.	normal				
2	Calsamate	25	7 s.i.d.	normal				
2	Acetylsalicylic acid	50	7 s.i.d.	normal				
2	Calsamate	50	7 s.i.d.	normal				
3	Acetylsalicylic acid	100	5 b.i.d.	1 mild; 1 medium; 1 severe				
3	Calsamate	100	5 b.i.d.					
2	Calsamate	100	11 b.i.d.	2 mild				
10	None	Controls	None	10 normal				
Microscopic pathology								
				lympho- cytic infiltra- tion	Capillary engorge- ment	Desqua- mation	Shed epithe- lium	Erosion of ulcer
5	Acetylsalicylic acid	50	23 s.i.d.	4	2	4	1	4
4	Calsamate	50	23 s.i.d.	2	0	2	0	1

dose had been administered the animals were killed by intravenous injection of a lethal dose of nembutal. Controls showed this had no effect on the gastric mucosa. Stomachs were removed at once with as little trauma as possible and opened along the lesser curvature. Gross observations revealed no definite pathological signs in any specimen. The results of histological studies on sections from the cardiac ring, pyloric ring, fundus, pyloric antrum, and junction of fundus with pylorus are summarized in table 2.<sup>2</sup>

<sup>2</sup> We wish to thank Dr. Norbert Enzer, Director of Laboratories, Mt Sinai Hospital, Milwaukee, for examining this material.





FIG. 4a. PHOTOGRAPH OF PYLORIC PORTION OF STOMACH OF A DOG FROM THE GROUP GIVEN ACETYLSALICYLIC ACID, 100 MG. PER KILOGRAM TWICE A DAY, SHOWING "MEDIUM" ULCERATION PRODUCED AFTER 5 DAYS OF SUCH TREATMENT  
Approximately  $\frac{1}{2}$  natural size



FIG. 4b. PHOTOGRAPH OF FUNDIC PORTION OF STOMACH OF A DOG FROM THE GROUP GIVEN CALSAMATE (EQUIVALENT TO 100 MG. ACETYLSALICYLIC ACID PER KILOGRAM) TWICE DAILY, SHOWING "MILD" ULCERATION PRODUCED AFTER 11 DAYS OF SUCH TREATMENT

There was no ulceration in animals after 5 days of treatment. Magnification approximately two times



As another test of gastric irritation the single doses of calsamate and acetylsalicylic acid which would induce vomiting were determined on 26 dogs. In general, each animal received each drug. No animal was used more than twice a week. Food was removed from the pens 4 to 6 hours before treatment. The drugs were given by stomach tube in 50 cc. of water per kilogram. The animals were observed continuously for 4 hours after medication. From table 3 it can be seen that for dogs the maximum tolerated dose of acetylsalicylic acid is between 75 and 100 mgm. per kilogram, since 8 of 14 animals

TABLE 3

*Incidence of vomiting in dogs given acetylsalicylic acid or calsamate (as the equivalent amount of acetylsalicylic acid); administered in 50 cc. of water per kilogram body weight*

DRUG	DOSAGE	NUMBER OF DOGS	NUMBER OF DOGS WHICH VOMITED
	mgm per kgm.		
Acetylsalicylic acid	600	8	8
Calsamate..	600	8	8
Acetylsalicylic acid	500	2	2
Calsamate	500	2	2
Acetylsalicylic acid	400	5	5
Calsamate	400	4	4
Acetylsalicylic acid	300	3	3
Calsamate . . .	300	3	2
Acetylsalicylic acid	250	10	10
Calsamate	250	10	3
Acetylsalicylic acid	200	6	4
Calsamate	200	6	1
Acetylsalicylic acid	100	14	8
Acetylsalicylic acid	75	9	1

vomited on administration of the higher dose and only one of 9 on the lower dose. The maximum tolerated dose of calsamate is 200 to 250 mgm. per kilogram, since emesis occurred in 3 of 10 instances on the higher dose and once in 6 animals with the lower dose. This vomiting dose of acetylsalicylic acid is lower than that given by Thompson and Dragstedt (1), who reported that 4 doses of 0.166 grams per kilogram at 30 minute intervals were required to cause emesis.

*Effect on kidney function and carbon dioxide combining power in the dog. In*



order to compare the effects of acetylsalicylic acid and calsamate on kidney function, urea clearance determinations were made on a group of 5 dogs by means of the technique previously described (10). After suitable controls two of the dogs received acetylsalicylic acid and 3 an equivalent amount of calsamate for 23 days. For the first 10 days the dose was 50 mgm. per kilogram daily, for the next eight days 100 mgm. per kilogram daily, and for the remainder of the experiment 100 mgm. per kilogram twice a day. Clearance determinations were made at weekly intervals.

Averages of the clearances before and after medication are given in table 4. Each group shows a higher average in the latter instance. In each case this is due to a marked increase by one animal of the group, amounting to 44 per cent for dog A following acetylsalicylic acid and 30 per cent for dog E following calsamate. There does not seem to be any significant difference

TABLE 4

*Urea clearance in dogs before and after acetylsalicylic acid and calsamate (as the equivalent amount of acetylsalicylic acid) (see text for dosages and duration of medication)*

DOG	DRUG	NUMBER OF CONTROL	CLEARANCES AFTER DRUG	AVERAGE OF CLEARANCES IN CC. PER M <sup>2</sup> PER MIN.	
				Control	After drug
A	Acetylsalicylic acid	9	9	45.6	65.7
B	Acetylsalicylic acid	6	10	38.3	44.7
Group average.....				41.7	55.2
C	Calsamate	6	9	37.1	40.8
D	Calsamate	6	9	52.5	45.0
E	Calsamate	6	9	48.0	62.4
Group average.....				45.9	49.4

between the two groups, the general tendency being toward an increased clearance.

Since Schnedorf, Bradley, and Ivy (6) previously had reported a tendency towards acidosis with prolonged administration of salicylates, plasma carbon dioxide combining power determinations were made at the same time that the urea clearance tests were performed. The method of Van Slyke and Cullen was employed (11). Under the conditions of these experiments neither drug caused an acidosis. Before acetylsalicylic acid the average control carbon dioxide combining power was 50.5 volumes per cent, and after medication the average for the two dogs was 48.8 volumes per cent. For the 3 dogs given calsamate the average control value was 50.3; and after medication, 50.2 volumes per cent.

*Toxicity studies.* The effects of repeated large doses of these two compounds were studied by giving one group of 10 rats 300 mgm. of acetylsalicylic



acid per kilogram daily for 10 days and another group of 10 animals the equivalent amount of calsamate for the same period of time. Autopsy on the eleventh day revealed no evidence of poisoning other than gastric effects previously described. Histological sections of liver and kidney showed no pathological changes in either group.

The acute maximum tolerated doses and the minimum lethal doses of these two drugs were determined on rats. The medication was given by stomach

TABLE 5

*Comparison of maximum tolerated and minimum lethal doses of acetylsalicylic acid and calsamate (as the equivalent amount of acetylsalicylic acid) in rats and rabbits*

NUMBER OF ANIMALS	DRUG	DOSAGE	FATE
Rats			
13	Acetylsalicylic acid	800	12 alive; 1 dead
2	Calsamate	800	2 alive
7	Acetylsalicylic acid	1,000	7 dead
13	Calsamate	1,000	13 alive
2	Acetylsalicylic acid	1,200	2 dead
7	Calsamate	1,200	7 dead
Rabbits			
4	Acetylsalicylic acid	400-600	4 alive
5	Calsamate	400-600	5 alive
8	Acetylsalicylic acid	800	8 alive
8	Calsamate	800	8 alive
8	Acetylsalicylic acid	1,000	5 alive; 3 dead
8	Calsamate	1,000	7 alive; 1 dead
12	Acetylsalicylic acid	1,200	6 alive; 6 dead
15	Calsamate	1,200	15 alive

tube in 5 cc. of water 12 hours after food had been removed from the cages. It can be seen from table 5 that for acetylsalicylic acid the maximum tolerated and minimum lethal doses are approximately 800 and 1000 mgm. per kilogram respectively, while the corresponding values for calsamate are 1000 and 1200 mgm. per kilogram respectively.

Further acute toxicity studies were carried out on rabbits. The animals were not fed for 24 hours before treatment. The drugs were administered



stomach tube in 100 cc. of water. The results of these experiments are so in table 5. As in the studies on rats, acetylsalicylic acid was more toxic than calsalumate. Six of 12 rabbits died following 1200 mgm. acetylsalicylic acid per kilogram, while all 15 animals survived a corresponding amount of calsalumate.

#### SUMMARY

In man the rate of urinary excretion of salicylic acid is essentially the same for calsalumate and acetylsalicylic acid, no matter whether the salicylates are given early or late after meals.

As determined by the dosages necessary to cause emesis in dogs and to produce gastric ulcers in rats, rabbits, and dogs, calsalumate causes less irritation of the gastric mucosa than does acetylsalicylic acid.

Neither calsalumate nor acetylsalicylic acid is shown to alter significantly renal clearances or plasma carbon dioxide combining power in dogs.

Calsalumate is less toxic than acetylsalicylic acid for rats and rabbits, since at the acute tolerated dose and the minimum lethal doses are higher for calsalumate than for acetylsalicylic acid.

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# THE EFFECT OF METRAZOL UPON THE BLOOD PRESSURE OF MAN AND DOG

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Received for publication August 12, 1941

The mechanisms which are responsible for the changes in the blood pressure following the injection of metrazol have been investigated only in cats (1, 2), dogs (3, 4) and rabbits (5). These earlier studies were made on anesthetized, decerebrated and spinal animals and showed that metrazol is capable of exciting the vasomotor and vagal centers. They do not show the blood pressure changes which are produced by the muscular contractions of the convulsions. Blood pressure studies in man following convulsive doses of metrazol are incomplete (for review of literature see Messenger and Moros (6)). Changes during the convulsion were not measured because the clinical (Korotkoff) method does not allow pressure determinations during convulsions, but immediately after the convulsions the arterial pressure was found either increased, decreased or unchanged. However, at this time any primary action of the metrazol is obscured by the many secondary effects either of the drug or of the convulsion.

In the present study continuous records of the femoral arterial pressure were obtained from five humans (patients with mental disorders) and six dogs. The use of the "hypodermic" manometer necessitated only local infiltration anesthesia. Convulsant doses of metrazol were rapidly injected intravenously in these humans and in dogs either without premedication or (on different days) after preliminary administration of curare, erythroidin, atropine, a spinal anesthetic or ergotoxine.

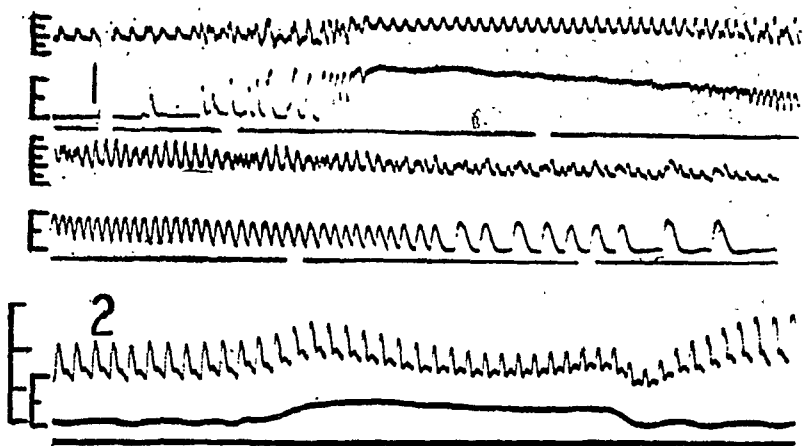
*Results in man.* A definite parallelism exists between the elevations of the arterial pressure and the muscular activity of the convulsions. As shown in figure 1, the arterial pressure suddenly rose with each seizure during the early clonic phase and the late clonic phase. It was elevated and remained high throughout the tonic phase.

These elevations of the arterial pressure, which frequently exceeded 100 mm. Hg, were accompanied by similar simultaneous rises in the intra-abdominal pressure (see figs 1 and 6). (The intra-gastric which differs only slightly from the intra-abdominal pressure was recorded from a balloon and



"evine" tube which were put through the nose, down the esophagus and to the stomach.) It has previously been shown during coughs and strains (8, 9, 10) that elevations of the intra-thoracico-abdominal pressure are transmitted to the heart and arteries in the thorax, abdomen and craniospinal and thereby elevate the arterial pressure.

The changes accompanying the convulsions, however, are not quite the same as those accompanying strains (compare fig. 1 with fig. 2). In prone



# 1. SIMULTANEOUS FEMORAL PRESSURE PULSES AND INTRA-GASTRIC PRESSURE (FROM ABOVE DOWNWARDS)

Tracings are from patient "B" without pre-medication. At the break in the record the upper left corner 6.8 cc. of metrazol (10 mgm. per kgm.) was injected intravenously. The tracings for the next 20 seconds were deleted. The first rhythmic clonic contractions produce similar pressure changes in the intra-abdominal (recorded as a-gastric) and arterial pressures. Throughout the tonic phase the intra-abdominal arterial pressures are elevated correspondingly. Again during the clonic phase, rise and fall in the intra-abdominal pressure causes a corresponding rise or fall to superimposed upon the arterial pulse. At the break in the record at the end of the clonic phase, tracings for 60 seconds are deleted. As shown the arterial pressure by that time has returned to the pre-injection values. Tracings during the next 30 minutes (presented) showed a 10 to 20 mm. Hg rise in arterial pressure for 5 minutes. In this and all subsequent records except where indicated differently, the pressure scales are given in units of 50 mm. Hg and the base line is interrupted at intervals of 10 seconds.

# 2. SIMULTANEOUS ARTERIAL PRESSURE PULSES AND INTRA-THORACIC PRESSURE FROM MAN

The signal indicates the duration of a strong prolonged strain

during strains the increase of the arterial pressure is very transient. The intra-thoracico-abdominal pressure elevates the arterial pressure but increases venous return to the right heart. As the pulmonary reservoir is filled with blood the arterial pressure and the pulse pressure decrease (7). During convulsions in man the main rise in the arterial pressure is also produced by the elevation of the intra-thoracico-abdominal pressure. However,



the arterial pressure and the pulse pressure increase during the tonic phase and early in the clonic phase. This occurs in spite of the fact that the intra-abdominal pressure may be decreasing slowly (see figs. 1 and 6). This rise in the net arterial pressure (arterial pressure minus intra-abdominal pressure) is rather small and may be the result of either an increased cardiac output or an increased peripheral resistance or both.

The pump-like action of muscular contractions increases venous return (11). This is clearly demonstrated during convulsions in dogs where peripheral pressures of 180 mm. Hg were recorded from a ligated femoral vein (see fig. 7). In the presence of an increased venous return, the absence of any significant rise in the pressure in the superior vena cava shows that the output of the right heart was increased. In man this increased output explains why the pulmonary reservoir is not depleted of blood during convulsions as is the case during the muscular activity accompanying prolonged strains.

The contracting muscles squeeze upon blood vessels over a large area of the body and reduce their size. This, and possibly vasoconstriction, increase the peripheral resistance which also contributes to the rise in the net arterial pressure.

The rôle played by vasoconstriction is shown by studies from three patients who received curare (Intocostin) and two who received erythroidin. These drugs produced marked asthenia and reduced the changes in the blood pressure which were secondary to the muscular contractions. The preparations used in this study had little if any effect upon the vasomotor system since they did not prevent the pressure rise of asphyxia (unpublished data). Yet large doses, which prevented the convulsions, definitely restricted the rise in arterial pressure (see fig. 3). Therapeutic doses, which merely reduced the severity of the convulsions, were somewhat less effective in preventing the rise of the arterial pressure (see fig. 4). Therefore this rise in blood pressure accompanied and, to a large extent, was produced by the convulsion.

A dose of metrazol (680 mgm.), which had regularly produced convulsions in a patient, failed during one experiment to produce the typical convulsions. The maximal rise in arterial pressure was only 26 mm. Hg. Apprehension was present and most of this small rise was probably psychic in origin.

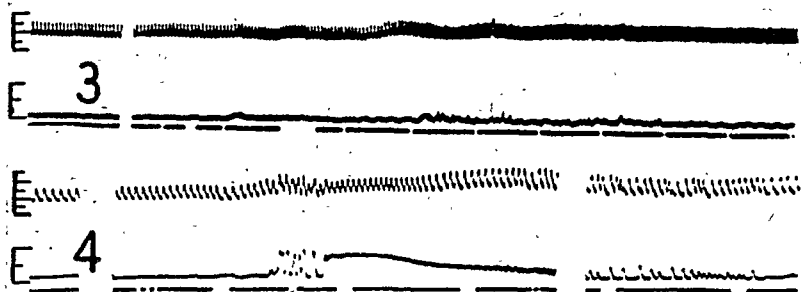
These data show that in man vasoconstriction plays only a minor part in the elevation of the arterial pressure produced by convulsant doses of metrazol. The large increase is dependent upon the presence of the convulsion.

In repeated experiments, while the arterial pressure was elevated during the convulsion, the heart rate always increased in three patients (average from 103 to 158 per minute) and always decreased in two patients (average from 108 to 60). At the end of the clonic phase the arterial pressure of one patient was still elevated (20/15 mm. Hg), but in the other four patients it was reduced below the pre-injection level (average reduction was 43/23 mm.



Fig). Within one minute after the end of the convulsions the arterial pressures of all of the patients had returned to, or was slightly above, the pre-convulsion values. Psychic effects and factors secondary to the convulsions count for many though not all of these changes in the heart rate and blood pressure. Some are evidently produced by the metrazol itself, because, as shown below, metrazol excites the parasympathetic and sympathetic nervous systems. The effects from this dual excitation of these systems mingle; those which predominate depend upon the individual patient and upon the type of pre-medication.

This dual excitation by metrazol is demonstrated in man by studies during thirteen convulsions while the patients were under the influence of spinal anesthesia where the sympathetic nerve roots are blocked, and during nine



FIGS. 3 AND 4. SIMULTANEOUS FEMORAL PRESSURE PULSES AND INTRA-GASTRIC PRESSURES FROM PATIENT "S"

Fig. 3. During the break in the record sufficient curare (13 cc. Intocostrin) was injected to produce pronounced asthenia, ptosis, inability to lift head from pillow, but not guttural speech. Signal indicates the injection of metrazol (13 mgm. per kgm.).

Fig. 4. During the first break in the record 11 cc. Intocostrin was injected. Signal indicates the injection of metrazol (12.5 mgm. per kgm.). Tracings during 30 seconds deleted at the second break in the record.

convulsions while the patients were under the influence of atropine sulfate where the parasympathetic endings were depressed.

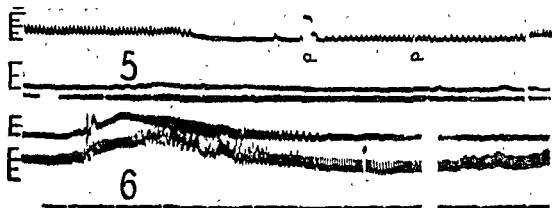
High spinal anesthesia ( $T_3$  to  $T_1$ ) limited the convulsions to the cephalic end of the body and blocked most of the sympathetic nerve roots. During convulsions the arterial pressure then was not increased but decreased to values such as 60/30 mm. Hg. The pressure returned to the pre-injection level only after the lapse of five to ten minutes. Tachycardia did not occur. In nearly two-thirds of the records bradycardia was present and lasted from 30 seconds to 4 minutes. This was extreme in one patient, who showed cardiac arrest for 17 seconds (see fig. 5). In this patient the spinal anesthesia extended to  $T_1$  and the procaine hydrochloride had blocked the cardiac accelerator and vasoconstrictor fibers. The extreme bradycardia and the small pulse pressure show that metrazol excites the parasympathetic system.



During a subsequent similar experiment premedication with 1 mgm. of atropine sulfate reduced the bradycardia. Larger doses of atropine were not tried, but should prevent this bradycardia.

In the presence of spinal anesthesia to T 7-T 9 metrazol produced small or insignificant changes in the arterial pressure. The net arterial pressure (arterial pressure minus intra-abdominal pressure) decreased during the tonic phase in spite of the increased heart rate. This indicates decreased peripheral resistance or decreased cardiac output.

The peripheral resistance is reduced by distention of blood vessels in the paralyzed areas, probably abetted by vasodilation brought about by unopposed parasympathetic excitation of these vessels.



FIGS. 5 AND 6. SIMULTANEOUS FEMORAL PRESSURE PULSES AND INTRA-GASTRIC PRESSURES

FIG. 7. Tracings of 40 seconds were deleted at the break in the record. (T-1). marked

the pressure bottle and show that the tracing is a tracing of 40 seconds were deleted at the break in the record.

FIG. 6. Tracings are from patient "T" who previously had received 0.8 mgm. of atropine sulfate. Signal indicates the injection of metrazol (13 mgm. per kgm.). Tracings of 30 seconds were deleted at the break in the record.

Decreased venous return limits the cardiac output. The skeletal muscles of the legs are paralyzed, those of the abdominal wall are weak or paralyzed and those of the thorax are capable of the usual activity. These conditions decrease venous return during the tonic convulsions due to the high intra-thoracic pressure.

When patients received therapeutic doses of atropine sulfate prior to the metrazol therapy, arterial pressures of 250 to 300 mm. Hg were commonly observed during the seizures in the clonic phase (see fig. 6). Depression of the cardio-inhibitors and vasodilators render them less active as compensatory mechanisms and the central excitation of them by the metrazol produces less response. The small increase in the net arterial pressure (25 to 30 mm. Hg) again emphasizes the minor rôle played by vasoconstriction.

As previously shown (7) the net arterial pressure is the pressure load



against which the left ventricle must work, and which is placed upon the arteries within the thoraco-abdominal-cerebrospinal cavities. The fact that convulsant doses of metrazol produce only small increases in the net arterial pressure in spite of large increase in gross arterial pressure means that the blood vessels of the vital areas (brain, heart and viscera) are not subjected to any large stresses during the convulsions.

*Results in dogs.* During the convulsions in unanesthetized dogs the intra-thoracic pressure was not elevated and the rise in the intra-abdominal pressure averaged less than 30 mm. Hg. Nevertheless, convulsant doses of metrazol elevated the arterial pressure 100 or even 200 mm. Hg. The return to the pre-injection pressure level was slow and required more than 10 minutes.

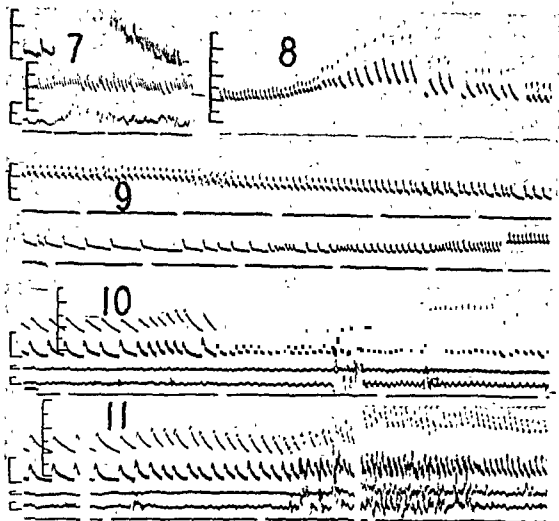
Several convulsions would occur after one large dose of metrazol (60 mgm. per kilogram). A large, prolonged rise of the arterial pressure occurred during and after only the first convulsion. In the succeeding convulsions the arterial pressure was elevated *only* during the convulsion and only 20 to 30 mm. Hg (see fig. 7). This corresponds to the small rise in the intra-abdominal pressure which occurs during the convulsions. In dogs an increase in the intra-thoraco-abdominal pressure makes only a minor contribution to the elevation of arterial pressure, whereas in humans it accounts for most of the rise in arterial pressure.

In dogs metrazol increases the arterial pressure by vasomotor excitation. After paralyzing doses of curare (3 mgm. per kilogram), convulsant doses of metrazol (15 to 20 mgm. per kilogram) still caused the same prolonged rise in arterial pressure (see fig. 8). This is not true in man (see fig. 3). The administration of sufficient ergotoxine ethanesulfonate (1.5 mgm. per kilogram) to give a reversal of action by epinephrine caused a reversal of action by metrazol. The prolonged reduction in the arterial pressure occurred during and after only the first convulsion when a large dose of metrazol was administered. In the succeeding convulsions from the same dose of metrazol there was no change in the net arterial pressure. These data show that convulsant doses of metrazol elevate the arterial pressure in man and in dogs by different mechanisms.

The increase in cerebral blood flow during and after convulsions observed in cats by Jasper and Erickson (12) may well be mainly passive to the rise in arterial pressure rather than from changes in the pH as was suggested by them. The fact that their data show that the increased blood flow accompanied only the first and not the subsequent convulsions and the fact that the increase in net pressure accompanied only the first and not the subsequent convulsions bear out this hypothesis.

Studies in dogs during spinal anesthesia prove that the parasympathetic nervous system is also excited by convulsant doses of metrazol as was previously reported (5). High spinal anesthesia (C-2) limited the convulsions to the head and neck muscles and paralyzed the sympathetic nervous system.





FIGS. 7-11. PRESSURE RECORDS FROM DOGS

superior vena cava was small indicates an

of dogs The scale of the superior vena

FIG. 8. Fc  
per kgm.).  
was injected  
injection of metrazol.

FIG. 9. Femoral pressure pulses from dog under the influence of spinal anesthesia (C-2). Five seconds prior to the start of the record 16 mgm per kgm of metrazol was injected. A continuous record is shown until the break at right of the second line where tracings of 90 seconds are deleted. Five minutes after the injection the arterial pressure again reached the pre-injection level.

FIG. 10. From above downwards, pressure pulses from carotid artery, pulmonary artery, pulmonary vein and superior vena cava of a dog lightly anesthetized with morphine sulfate and pentobarbital-sodium. At signal 25 mgm per kgm. of metrazol was injected, which in this lightly anesthetized dog caused extensive muscle twitches for two seconds but failed to cause convulsions. The pressure scales of the pulmonary vein and superior vena cava are shown in units of 10 mm. Hg

FIG. 11. Pressure records from the same blood vessels and from the same animal as those, in fig. 10. These were recorded twenty minutes later than those in fig. 10. During the first break in the record a convulsive dose of metrazol (30 mgm. per kgm.) was injected and tracings of the next 10 seconds were deleted. At the second break tracings for 30 seconds are deleted and pressure pulses during the last part of the clonic phase are then presented. The convulsions produced vibrations in the leaden tubes



With the onset of convulsions the arterial pressure decreased and returned to the pre-injection value only after three minutes (see fig. 9). Towards the end of the tonic phase and in the clonic phase bradycardia was present for 15 to 30 seconds. Since metrazol does not produce vasodilation in perfused organs (5), the initial reduction in arterial pressure is evidently caused by vasodilation from central excitation and not by peripheral action (4). The bradycardia also results from vagal excitation by the metrazol. These vagal effects were disclosed when the sympathetic nervous system was blocked by the procaine hydrochloride.

Subconvulsant doses (8 mgm. per kilogram) elevated the arterial pressure 10 mm. systolic and 25 mm. diastolic without producing any initial reduction in the blood pressure. These results differ from those previously reported from anesthetized animals (dogs (4) and rabbits (5)), where doses of 5 to 10 gm. per kilogram lowered the arterial pressure 15 to 20 mm. Hg. It appears that anesthesia and operative procedures reduce the effects of the sympathetic excitation produced by metrazol.

*Pulmonary pressures in dogs.* Previous studies (13, 14, 15) have shown that the vasomotor mechanisms of the pulmonary circulation of dogs are not significantly influenced by cardio-vascular drugs such as epinephrine, the nitrites, methyl acetylcholine and histamine.

In the present study simultaneous pressure records were obtained from the pulmonary artery, pulmonary vein, a systemic artery and the superior vena cava of one unanesthetized and two anesthetized dogs (for description of methods see references 13, 14, 15). As shown in figures 10 and 11, subconvulsant and convulsant doses of metrazol produce only a slight rise in the pulmonary arterial pressure and very little change in the pulmonary pressure gradient (pulmonary arterial pressure minus pulmonary venous pressure). Pulmonary vaso-constriction, if present, is certainly ineffective in the dog.

During metrazol convulsions the contractions of skeletal muscles, together with vasoconstriction in dogs, reduce the vascular area of the systemic circuit. One part of the compensatory mechanism is a shift of blood to the pulmonary reservoir. This explains the small pressure rise which is present in records from the pulmonary artery and the pulmonary vein of dogs. In man the intra-thoracic pressure is increased during the convulsions. The shift of blood to the pulmonary reservoir would be expected to occur only after the superficial veins become distended.

#### SUMMARY AND CONCLUSIONS

The species variation is pronounced with respect to the effect of metrazol upon the arterial pressure of man and dog.

In man convulsant doses of metrazol increase the arterial pressure by an average maximum of 100 mm. Hg. Vasoconstriction plays only a minor role. The main rise is limited to the duration of and is produced by the contractions of the skeletal muscles.



In man these contractions increase markedly the extravascular pressure in most areas of the body, squeeze the blood vessels and elevate the gross arterial pressure, but do not increase the effective pressure to the vital areas (brain, heart and viscera). The blood vessels of these vital areas are not subjected to any additional stress.

During the first minute immediately after the convulsion, the arterial pressure in four of the five patients was definitely reduced below the pre-injection value.

In man metrazol excites the parasympathetic and sympathetic nervous systems. The effects upon the arterial pressure and the heart rate are mingled. Those, which predominate, depend upon the type of premedication and upon the individual patient. In the presence of high spinal anesthesia metrazol can produce dangerous degrees of bradycardia. This can be decreased by therapeutic doses of atropine sulfate.

The use of curare (16) or erythroidin (17) to protect the patient against excessive stresses caused by the convulsion is definitely superior to the use of high spinal anesthesia (18).

In unanesthetized dogs subconvulsant and convulsant doses of metrazol produce large prolonged elevations of the arterial pressure. In contrast to man, these elevations in dogs are produced by vasoconstriction. Parasympathetic excitation also occurs, but is effectively masked unless the sympathetic system is rendered ineffective by high spinal anesthesia or ergotoxine. As in man the convulsions themselves increase venous return and peripheral resistance, but they increase the arterial pressure only 20 or 30 mm. Hg. This is because the convulsions in dogs fail to produce a large increase in the intra-thoraco-abdominal pressure. Consequently the large arteries and the blood vessels of the viscera are subjected to very little extra-vascular pressure.

Metrazol like other vasomotor drugs has very little effect upon the pressure gradient through the pulmonary circulation of dogs. Pulmonary vasoconstriction, if present, is certainly ineffective in the dog.

We are indebted to E. R. Squibb and Sons for the curare (intocostin), to Merek and Co. for the  $\beta$ -erythroidin hydrochloride and to Billhuber, Knoll Corp. for the metrazol used during these studies.

Aid from a grant of the American Medical Association is gratefully acknowledged.

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# THE EFFECT OF CERTAIN ANESTHETICS ON BLOOD KETO ACID LEVELS<sup>1</sup>

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Received for publication August 14, 1941

In the course of studies reported previously (1) on shock induced by hemorrhage, we found the level of blood keto acids to be elevated in this condition, and that thiamin would reduce these elevated values. We found this phenomenon also to occur in animals anesthetized with ether but not in dogs anesthetized locally with procaine solution. It was thought desirable to determine whether or not the same elevation of blood keto acids occurred in animals anesthetized with other anesthetics, namely cyclopropane, pentothal sodium, pentobarbital sodium, and propazone sodium (5,5-Di-*n*-propyl-2,4-oxazolidinedione) (2).

*Methods.* Dogs were used as experimental animals. All blood samples were drawn by venipuncture from the femoral veins.

Blood keto acids, which probably include pyruvic, aceto-acetic, oxaloacetic, and  $\alpha$ -ketoglutaric, were determined by the method of Lu (3) with the modification of Bueding and Wortis (4), (using 3*N* NaOH). This method as used by us has been found accurate to five per cent.

## EXPERIMENTS

### Group I

Table 1 shows the keto acid values obtained in eight dogs under diethyl ether anesthesia which was maintained at the surgical level by tracheal cannula and ether bottle.<sup>2</sup>

Six of the eight dogs showed a significant rise in blood keto acids, the remaining two exhibiting no significant change.

After three hours of anesthesia, thiamin chloride was administered to five of these dogs in the dosage shown on the table. In the four out of five dogs whose keto acids were elevated, thiamin resulted in a gradual lowering of these values. Thiamin chloride was administered as a solution in distilled water containing 10.0 mgm. per cubic centimeter.

<sup>1</sup> This work was aided by grants from the Mallinckrodt Chemical Works and the National Research Council.

<sup>2</sup> Data for this group of animals have been reported in a preceding publication (1).



It was thought desirable to determine the length of time necessary for the elevated keto acid level to return to normal after ether administration was stopped, without administration of thiamin. Two dogs were anesthetized for two hours with ether administered through a Guedel tracheal catheter.

TABLE 1  
*Ether—keto acids*  
Results in mgm. per 100 cc.

	DOG NUMBER							
	I Keto acids	II Keto acids	III Keto acids	IV Keto acids	V Keto acids	VI Keto acids	VII Keto acids	VIII Keto acids
Control.....	2.3	1.9	1.8	2.0	2.1	2.4	2.3	2.6
15 minutes later.....			1.9	2.2	2.0			2.3
Ether begun								
1 hour.....	3.0	2.6	2.0	2.9	2.2	2.3	2.5	2.8
2 hours.....	3.6	3.6	1.7	2.9	3.5	2.6	3.3	3.9
3 hours.....	3.5	3.6	1.7	3.5	3.4	2.0	3.0	4.1
	*	*	*	*	*			
4 hours.....	2.3	3.1	1.9	3.4	3.1	2.1	3.3	
5 hours.....	2.3	2.7	1.7	3.1	3.1	2.4	3.2	
6 hours.....	2.1	2.4	2.1	2.7	2.8	2.6	3.4	
7 hours.....			2.0	2.2	2.7			
8 hours.....			1.7	2.3	2.8			

\* Thiamin: intravenously 1 mgm. per kgm. IV and 2 mgm. per kgm. intramuscularly, then 0.5 mgm. per kgm. intramuscularly every hour thereafter.

TABLE 2  
*Cyclopropane—keto acids*  
Results in mgm. per 100 cc.

	DOG NUMBER					
	I Keto acids	II Keto acids	III Keto acids	IV Keto acids	V Keto acids	VI Keto acids
Control.....	2.5	2.4	2.5	2.2	2.5	3.0
Control 15 minutes later.....			2.3	2.1	2.4	2.7
Cyclopropane begun						
1 hour.....	2.3	2.3	2.4	2.0	2.6	2.8
2 hours.....	2.2	2.1	2.2	3.3	2.6	2.6
3 hours.....	2.1	2.8	2.1	2.5	2.4	
4 hours.....	1.8	3.1	2.5	2.6	2.0	

It was found that in both cases the elevated keto acid level had returned to normal two hours after the ether bottle was removed.

### Group II

Table 2 shows the values obtained from six dogs under closed anesthesia with cyclopropane.



Two of the six animals exhibited a rise in blood keto acids, two a fall, and two no change. In dog Number IV, the anesthesia was deepened to the stage of respiratory arrest a few minutes before the fourth blood sample was drawn.

TABLE 3  
*Pentobarbital—keto acids*  
Results in mgm. per 100 cc.

	DOG NUMBER	
	I Keto acids	II Keto acids
Control	2.2	2.2
Control 15 minutes later	2.1	2.2
Pentobarbital begun		
1 hour	1.9	1.7
2 hours	1.5	1.6
3 hours	1.5	1.5
4 hours	1.5	1.5

TABLE 4  
*Pentothal—keto acids*  
Results in mgm. per 100 cc.

	DOG NUMBER			
	I Keto acids	II Keto acids	III Keto acids	IV Keto acids
Control	2.6	3.1	1.9	2.2
Control 15 minutes later		3.1	1.9	
Pentothal begun				
1 hour	1.8	2.0	1.6	2.0
2 hours	1.8	2.0	1.5	2.6
3 hours	1.7	1.5	1.6	2.3
4 hours	1.8	1.6	1.6	2.0

TABLE 5  
*Propazone—keto acids*  
Results in mgm. per 100 cc.

	DOG NUMBER			
	I Keto acids	II Keto acids	III Keto acids	IV Keto acids
Control	1.7	1.4	2.1	2.1
Control 15 minutes later	1.5	1.2		2.1
Propazone begun				
1 hour	1.6	2.0	1.5	2.1
2 hours	1.1	1.1	1.5	1.9
3 hours	1.3	1.4	1.5	1.9
4 hours	1.1	1.3	1.1	2.0



This was followed by a rise in blood keto acids of 1.3 mgm. per 100 cc., apparent in the next sample.

### *Group III*

In table 3, two dogs are seen to show no rise in blood keto acid values during four hours of anesthesia with pentobarbital sodium.

### *Group IV*

The dogs of this group, anesthetized for four hours with pentothal sodium, showed either no significant change or a fall in keto acid levels. These results may be seen in table 4.

### *Group V*

We were interested to see whether a non-barbituric acid hypnotic would produce a keto-acidosis, and inasmuch as Stoughton and Baxter (2) have shown that 5,5-Di-*n*-propyl-2,4-oxazolidinedione produces no significant change in blood sugar, this drug was tried. As may be seen in table 5, no keto acidosis occurred with this substance.

## DISCUSSION

Platt and Lu (5) have shown that the increase of blood pyruvate values may be correlated with the degree of vitamin B<sub>1</sub> deficiency in cases of beri-beri, and that the blood pyruvate determination may be useful as a diagnostic test in this disease. It is well known that the need for thiamin as cocarboxylase depends on the amount of carbohydrate metabolized. Ether anesthesia has been shown by several investigators (6, 7) to produce hepatic glycogenolysis, hyperglycemia, hyperlactacidemia, and a rise in blood acetone bodies. Thus, in view of the high blood sugar seen in these animals, an increased need for thiamin, as shown by a piling-up of blood keto acids and among them pyruvic acid, is not surprising. That this hypothesis may be tenable is shown by the reduction in keto acid values occurring after administration of thiamin to these dogs.

Cyclopropane has been shown to produce a mild hyperglycemia by Henderson and Lucas (8) and others (9). That this rise in blood sugar should cause accumulation of keto acids in an occasional animal which may be slightly bi-avitaminotic, may be expected.

Pentothal was chosen as an example of a thiobarbiturate since it is becoming widely used for short anesthesia. It has been shown to produce no hepatic glycogenolysis by Reindollar (10). Pentobarbital sodium has been shown to prevent either hyperglycemia by Campbell and Morgan (11). No pyruvic acidosis would be expected from these barbiturates, and we have shown that an actual fall in keto acid values occurs.



## SUMMARY

1. A rise in blood keto acid levels may occur in dogs anesthetized with diethyl ether and in those anesthetized with cyclopropane.

2. Elevated keto acid values occurring in dogs under ether anesthesia may be reduced by administration of thiamin:

3. Pentothal-sodium, pentobarbital sodium, and 5,5-Di-*n*-propyl-2,4-oxazolidinedione produce no elevation in blood keto acid values.

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# HYPNOTICS AND BLOOD SUGAR REGULATING CENTRES

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Received for publication May 20, 1941

It has been established that the striking antidotal properties of picrotoxin in barbiturate poisoning are due to a reciprocal pharmacological mechanism (18, 9, 10, 11, 8, 7). Picrotoxin produces an awakening effect in barbiturate sleep and is now used for the treatment of acute barbiturate poisoning; barbiturates can protect against several times the lethal dose of picrotoxin. Further aspects of this antagonism were investigated by Das (2) and Rosenthal (15). Besides its stimulating action on the vagal centres picrotoxin also stimulates sympathetic centres, and in subconvulsant doses produces a marked hyperglycemia of central origin. It does not produce hyperglycemia after double splanchnicotomy or denervation of both adrenal glands. It does not affect the blood sugar level after section of the spinal cord at the level of the 7th and 8th dorsal segment or after the administration of ergotamine (14).

This paper deals with the effect and mode of action of aliphatic hypnotics on the blood sugar regulating centres when the latter had been stimulated by picrotoxin. According to Pick (12) the hypnotics can be divided into two groups, the "cortical," which act chiefly by depressing the cerebral cortex (e.g. paraldehyde and chloral hydrate) and the "thalamic" which act by depressing the centres in the thalamic region, (e.g. the derivatives of barbituric acid, chlorotone, urethane). Vidal (17) found that intravenous injection of somnifen prevents the glycosuria due to Bernard's puncture; this is not the case with urethane. Amidopyrin hyperglycemia proved to be of central origin and can be suppressed by barbital and phenobarbital.  $MgSO_4$  hyperglycemia is suppressed by chloral hydrate but not by barbiturates (6). According to Tachinaba (16) barbital and phenobarbital annul morphine, conitine and picrotoxin hyperglycemia, whereas chloral hydrate and urethane increase it.

**METHODS.** Hagedorn-Jensen's method was used for the determination of the blood-sugar in rabbits. One milligram of picrotoxin per kg. injected subcutaneously regularly produced hyperglycemia within 2-3 hours; the convulsant and often fatal dose is 1.4-1.5 mgm. per kilogram. The rabbits were fed with a mixture of oats, bran and the vegetables available at the time. The hypnotics were administered either at the same time as the picro-



toxin injection or at the intervals mentioned in the tables. Experiments and controls were mostly carried out on the same animals (cf. Friedberg (4)).

RESULTS. 1. *Depression of blood sugar regulating centres by hypnotics.* Picrotoxin hyperglycemia is abolished by either group of hypnotics in nar-

TABLE 1  
*Blood sugar (in mgm. per 100 cc.)*

TIME	A PARALDEHYDE			B CHLORAL HYDRATE		
	Paraldehyde + picrotoxin	Control experiments		Chloral hydrate + picrotoxin	Control experiments	
		Picrotoxin	Paraldehyde		Picrotoxin	Chloral hydrate
Before the experiment . . . .	98	82	89	107	133	84
1 hour after picrotoxin injection . . . . .	104	248	129	122	210	92
2 hours . . . . .	113	264	138	102	200	114
3 hours . . . . .	123	205	96	96	168	105

Suppression of picrotoxin hyperglycemia in rabbits (1 mgm. of picrotoxin per kilogram subcutaneously) by: A, paraldehyde (2 cc. per kilogram orally, diluted with 50 cc. of water); B, chloral hydrate (0.4 gram per kilogram orally, diluted with 50 cc. of water).

TABLE 2  
*Blood sugar (in mgm. per 100 cc.)*

TIME	A. PHENOLBARBITAL			B CHLORETONE		
	Phenobarbital + picrotoxin	Control experiments		Chloretone + picrotoxin	Control experiments	
		Picrotoxin	Pheno- barbital		Picrotoxin	Chloretone
Before the experiment . . . .	121	126	112	121	109	118
1 hour after picrotoxin injection . . . . .	143	232	128	116	187	122
2 hours . . . . .	122	253	139	132	278	139
3 hours . . . . .	117	158	121	133	259	113

Suppression of the picrotoxin hyperglycemia (1.2 mgm. of picrotoxin per kilogram subcutaneously) by: A, phenobarbital (0.08 gram per kilogram subcutaneously); B, chloretone (2 cc. of 10 per cent chloretone in 50 per cent alcoholic solution per kilogram orally, diluted with 50 cc. of water).

cotic doses. Tables 1 and 2 illustrate the results of representative experiments with the "cortical" hypnotics, paraldehyde and chloral hydrate, and the "thalamic" hypnotics, phenobarbital and chloretone (22 experiments).

Picrotoxin hyperglycemia is however not prevented even by large doses of



urethane which is classified by Pick as a thalamic hypnotic (6 experiments). This is well seen in table 3.

In experiment A, 1.5 gm. of urethane per kilogram in spite of a marked narcotic action did not prevent the picrotoxin hyperglycemia which took about the same course as in the control experiment. As is well known, urethane by itself can produce a hyperglycemia which usually begins to develop some hours after the treatment and continues for 24 hours and more. It differs in this way from other kinds of hyperglycemia of central or peripheral mechanism (1, 13). Experiment B demonstrates such a hyperglycemia 20 hours after administration of 1.5 gm. of urethane. The additional dose of 1.0 gm. of urethane per kilogram did not suppress a further increase of the blood sugar level by picrotoxin.

TABLE 3  
*Blood sugar (in mgm. per 100 cc.)*

TIME	A			B		
	Urethane + picrotoxin	Control experiments		Urethane + picrotoxin	Control experiments	
		Picrotoxin	Urethane 1.5 grams per kilogram		Picrotoxin	Urethane 1.0 gram per kilogram
Before the ex- periment:.....	139	121	114	225	130	136
Four after pic- rotoxin injec- tion.....	209	226	99	314	260	148
Hours.....	221	239	109	332	223	124
Hours.....	178	89	109	174	175	154

Occurrence of picrotoxin hyperglycemia (1.2 mgm. of picrotoxin per kilogram subcutaneously) during the hypnotic action of urethane: A, 1.5 grams urethane per kilogram orally in 50 cc. of water; B, 1st column, urethane hyperglycemia 20 hours after oral administration of 1.5 grams urethane per kilogram; then 1.0 grams urethane per kilogram orally in 50 cc. of water and 1.2 mgm. of picrotoxin per kilogram subcutaneously.

Although the hypnotics in larger doses can have peripheral paralyzing effects on various organs (cf. 5), the suppression of the picrotoxin hyperglycemia by the aliphatic hypnotics mentioned is probably entirely due to depression of the blood sugar regulating centres. This view is supported by the fact that the peripheral glycogenolytic effect of adrenaline is not annulled by chloral hydrate, paraldehyde, barbital, chloretone (12 experiments). Table 3 demonstrates the occurrence of adrenaline hyperglycemia in paraldehyde and chloretone sleep.

On the other hand urethane in spite of its narcotic action does not inhibit the central glycogenolytic action of picrotoxin. This shows that the narcotic effect of hypnotics can sometimes take place without the blood sugar regulating centres being simultaneously affected.



2. *Sensitiveness of the blood sugar regulating centres to hypnotics.* Although in the experiments described above the animals were awakened by the injection of picrotoxin the depression of the sugar centres produced by hypnotics was not abolished. This suggests that the sugar centres are readily affected by many hypnotics and that there is no parallelism between the awakening mechanism of picrotoxin and its stimulating action on the sugar centres.

The marked sensitiveness of the sugar centres to many hypnotics is confirmed by the following experiments. Smaller doses of hypnotics without any detectable influence either on the sleep centres or on the temperature centres may annul the stimulating action of picrotoxin on the sugar centres; 0.75 cc. paraldehyde per kilogram orally, or 0.03 gm. per kilogram of phenobarbital subcutaneously, suppressed the picrotoxin hyperglycemia although

TABLE 4  
*Blood sugar (in mgm. per 100 cc.)*

TIME	A. PARALDEHYDE			B. CHLORETONE		
	Paraldehyde + adrenaline	Control experiments		Chloretone + adrenaline	Control experiments	
		Adrenaline	Paraldehyde		Adrenaline	Chloretone
Before the experiment	121	114	89	108	110	103
1 hour after adrenaline injection . . .	304	319	143	305	245	112
2 hours . . . .	326	340	129	344	242	119
3 hours . . . .	216	237	96	105	149	89

Occurrence of adrenaline hyperglycemia (0.3 mgm per kilogram subcutaneously) during the hypnotic action of: A, paraldehyde (2 cc. per kilogram orally, diluted with 50 cc. of water), B, chloretone (2 cc. of 10 per cent chloretone in 50 per cent alcoholic solution per kilogram orally, diluted with 50 cc. of water).

they did not produce sleep or a fall of body temperature. The injection of 0.03 gm. of barbital per kilogram, though it produced no effect on sleep or body temperature, prevented for at least six hours the development of the picrotoxin hyperglycemia (7 experiments).

The depression of the sugar centres by hypnotics may outlast the spontaneous awakening by many hours. The re-awakening of the sugar centres measured by means of their response to picrotoxin (1.2 mgm. per kilogram) was systematically followed after the injection of 0.1 gm. of barbital and 0.4 gm. of chloral hydrate per kilogram (8 experiments). A representative experiment is reproduced in table 5.

Twelve hours after the injection of barbital the sugar centres were still depressed and did not react to picrotoxin, but by then the animals were wide awake again, and the body temperature had spontaneously returned to nor-



ial. Not until twenty hours after the barbital treatment did the sugar centres regain their normal reactivity to picrotoxin.

In experiments with 0.4 gm. per kilogram of chloral hydrate orally spontaneous re-awakening took place two or three hours after administration, but at that time the sugar centres did not yet respond to 1.2 mgm. of picrotoxin per kilogram subcutaneously.

3. *The awakening effect of larger doses of picrotoxin on the narcotised sugar centres.* The single dose of hyponotics which would just cause deep sleep was examined in preliminary experiments. It was defined as the smallest doses of a hypnotic producing sleep accompanied by loss of muscular tonus and the adoption of a passive side position by the rabbits. As mentioned, this minimum dose varies in different animals; it was therefore determined in the same rabbit as those on which the main experiments were performed. Picrotoxin injections were repeated every hour until an increase of the

TABLE 5  
*Blood sugar (in mgm. per 100 cc.)*

TIME	INTERVAL BETWEEN BARBITAL AND PICROTOXIN INJECTION			CONTROL EXPERIMENTS	
	6 hours	12 hours	20 hours	Picrotoxin	Barbital
Before the experiment.	124	114	86	101	122
1 hour after picrotoxin injection.....	114	98	212	226	144
2 hours.....	132	112	210	270	116
3 hours.....	116	118	144	249	146

Reappearance of the picrotoxin hyperglycemia (1.2 mgm. per kilogram subcutaneously) after narcosis of the sugar centres by 0.1 gram of barbital per kilogram subcutaneously.

Blood sugar level occurred. Whereas in the normal rabbit 1.0 mgm. of picrotoxin causes a marked hyperglycemia, the sugar centres under the influence of barbital begin to react only to six times this amount of picrotoxin injected in divided doses over a period of four hours. Even with this dose, always fatal in the normal rabbit, the hyperglycemia is less marked than that occurring in the normal rabbit after 1 mgm. of picrotoxin. This hyperglycemia produced five hours after the repeated picrotoxin injections cannot be attributed to spontaneous recovery of the sugar centres but is due to an active abolition of the narcosis of the sugar centres; for, as table 5 demonstrates, the sugar centres do not respond even twelve hours after a barbital injection to a single injection of 1.2 mgm. of picrotoxin. Therefore the sensitiveness of the sugar centres after administration of 0.1 gm. of barbital is about six times less than of the normal sugar centres.

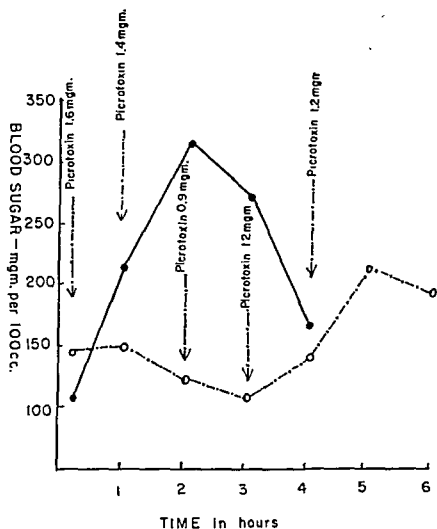
By comparison with the effect of this thalamic hypnotic, the narcosis of



the sugar centres by the cortical hyponotics chloral hydrate and paraldehyde can be overcome more easily by picrotoxin. A dose of 0.4 gm. of chloral hydrate per kilogram suppresses the effect of 1.3 mgm. picrotoxin, but 1.6

TABLE 6

*Forced interruption of the narcosis of the sugar centres (produced by 0.1 gram barbitone per kilogram subcutaneously) by large doses of picrotoxin*



● — ● — ●: Blood sugar curve in a normal rabbit after the injection of 1 mgm. of picrotoxin per kilogram subcutaneously.

○ . . . . ○ . . . . ○: Blood sugar curve in the same animal under the influence of 0.1 gram of barbitone per kilogram after the administration of 6.3 mgm. picrotoxin per kilogram, subcutaneously, injected in divided doses over a period of four hours.

mgm. of picrotoxin can produce a marked hyperglycemia. This shows that the sensitiveness of the sugar centres to picrotoxin under the action of chloral hydrate is only 1.6 times less than that of the normal sugar centres.

Two cubic centimeters of paraldehyde per kilogram orally inhibit the effect

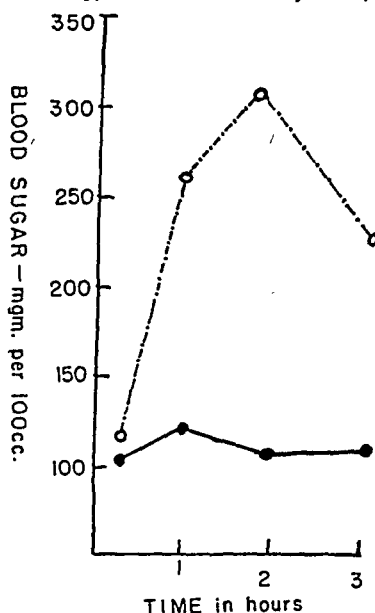


1 mgm. and 1.4 mgm. of picrotoxin on the sugar centres, but 1.9 mgm. of picrotoxin produce a marked hyperglycemia. Therefore the sensitivity of the sugar centres to picrotoxin under the depressing action of paraldehyde is about 1.9 times less than that of the normal sugar centres.

A comparison between these results suggests that the thalamic hypnotic chloral hydrate has a stronger depressing action on the blood sugar regulating

TABLE 7

Interruption of the narcosis of the sugar centres (produced by 0.4 gram of chloral hydrate per kilogram orally, diluted with 50 cc. of water) by picrotoxin



● — ● — ●: Blood sugar curve in a rabbit under the simultaneous action of chloral hydrate and 1.3 mgm. of picrotoxin per kilogram subcutaneously.

○ - - - ○ - - - ○: Blood sugar curve in the same animal under simultaneous action of chloral hydrate and 1.6 mgm. of picrotoxin per kilogram cutaneously.

... differs than the cortical hypnotics chloral hydrate and paraldehyde. Differences in the rate of excretion and destruction cannot play a decisive part here, because the chloral hydrate and paraldehyde narcosis of the sugar centres is interrupted by picrotoxin within the first hour after beginning experiments.

DISCUSSION. The evidence presented above shows that many hypnotics, with the exception of urethane can depress the blood sugar regulating centres.



Like the hypothalamic centres for water-regulation and the centres in the corpora quadrigemina for the coordination of the vomiting mechanism (Pick and coworkers), they can be depressed even by doses which do not produce sleep; like the powerful action of phenobarbital on the diuresis centre, the excitability of the blood sugar centres can also be suppressed by barbiturates for many hours. This long lasting effect is in accordance with observations of Biehler (1935) that barbital even seven days after its administration can inhibit twice the convulsive dose of metrazol.

#### SUMMARY

1. Picrotoxin hyperglycemia is regularly suppressed by many aliphatic hypnotics such as chloral hydrate, paraldehyde, barbiturates and chloretone. This effect is produced by a depressant action on the blood sugar regulating centres.

2. Urethane even in larger doses cannot annul the central glycogenolytic action of picrotoxin. Therefore hypnotic action can take place without the sugar centres being involved.

3. The depression of the sugar centres by hypnotics can take place independently of any action on the sleep mechanism. They can be depressed by smaller doses of hypnotics which do not produce sleep, and the depression of the sugar centres can outlast awakening by many hours.

4. Barbiturates have a stronger depressing action on the sugar centres than chloral hydrate and paraldehyde. In equivalent hypnotic doses barbital diminishes the sensitivity of the sugar centres to picrotoxin about six times, but chloral hydrate and paraldehyde only 1.6 to 1.9 times.

I am indebted to Mr. F. L. Attenborough, M.A., Principal of the University College, Leicester, for the hospitality given to me, and Miss A. Hosker, Ph.D., for her kind help during the experiments. I am grateful to Professor S. Wright for his revision of the manuscript.

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# ACTION OF DRUGS BENEFICIAL IN MYASTHENIA GRAVIS

## I. EFFECT OF PROSTIGMINE AND GUANIDINE ON SERUM AND MUSCLE POTASSIUM

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Received for publication August 28, 1941

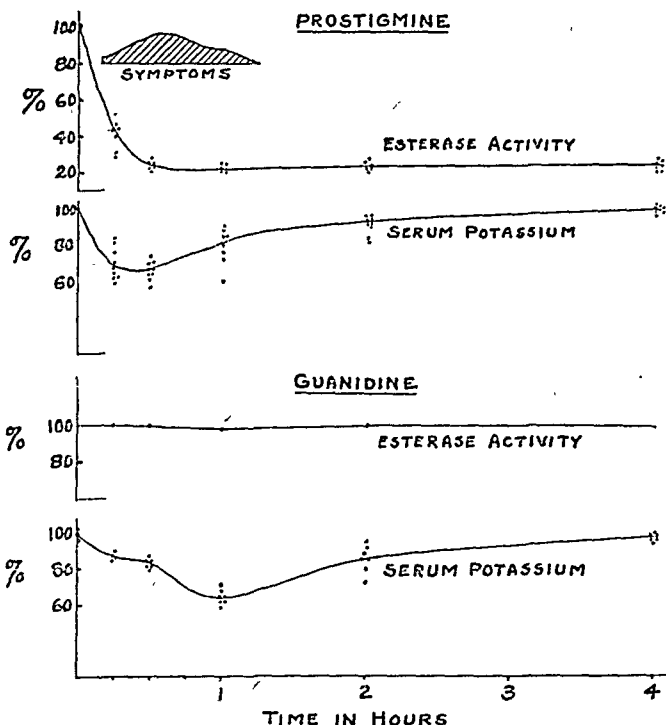
The commonly accepted theory of the mode of action of prostigmine is that it inhibits acetylcholine esterase. However, in recent years, considerable evidence has accumulated indicating that this is by no means the only action of the drug. One of the outstanding therapeutic uses of prostigmine has been in the treatment of Myasthenia gravis. Other drugs (i.e. guanidine, ephedrine and potassium chloride) are also efficacious in the treatment of this condition. These remedies have no effect on the acetylcholine esterase; in fact, no common basis of action has been suggested for these drugs. It is our object, in commencing these investigations, to attempt to discover some common mode of action by which these remedies may produce their beneficial effects. Potassium has long been suspected to be of fundamental importance in physiological processes, although its exact mode of action in muscular contraction is unknown (1). Cummins (2) reported changes in serum and muscle potassium after the administration of prostigmine to myasthenic patients. Potassium chloride has proven helpful in certain of these cases. Harvey (3) demonstrated increased sensitization of isolated frog muscle to potassium chloride after guanidine. It therefore seemed important to determine the effect of prostigmine and guanidine on potassium distribution in blood and muscle.

**METHODS.** Serum potassium in humans and dogs was determined by the method of Breh and Gaebler (4), in rats by the method of Wretling (5). Potassium in muscle was estimated by Cummins' (6) procedure. The dogs and rats were full grown stock animals, selected at random. Acetylcholine esterase activities were measured by the titration method of Hall and Lucas (7).

**RESULTS.** 1. *Effect of prostigmine and guanidine on serum potassium.* In dogs prostigmine was given subcutaneously in doses of 1 mgm. regardless of weight. Guanidine carbonate (20 mgm. per kilogram) was dissolved in saline and administered intravenously. Both of these drugs lowered serum potassium in dogs (fig. 1). The maximum effect of prostigmine was reached in 30 minutes, while that of guanidine required 60 minutes. The symptoms



duced by prostigmine rarely lasted longer than 60 minutes after injection the drug and the maximum reactions were usually seen about 30 minutes after administration. In some animals, the prostigmine reaction was so severe that atropine had to be administered. This did not prevent the fall in serum potassium.



#### 1. EFFECT OF PROSTIGMINE AND GUANIDINE ON SERUM POTASSIUM AND ESTERASE ACTIVITY IN DOGS

The drugs were injected immediately after the initial blood sample was drawn. Additional blood samples were taken at the times indicated, and esterase activities and serum potassiums determined. Symptoms of prostigmine activity were evaluated from relative amount of salivation, lacrimation, nasal discharge and muscular tremors.

The acetylcholine esterase activity has also been followed after these two drugs (fig. 1). By our technique guanidine produced no inhibition of the esterase. Prostigmine, on the other hand, effected a prolonged decrease in esterase activity, the duration of this action being much longer than the signs of prostigmine activity and the lowering of the serum potassium.

In rats, serum potassium was likewise lowered. Because of the size of the animal and amount of blood required for analysis, the determinations were



limited to one post-injection sample. With 0.05 mgm. prostigmine (subcutaneously), the serum potassium fell to 61 to 68 per cent (three rats) of the initial level within 15 minutes after injection. After guanidine (30 mgm. of the carbonate subcutaneously) the serum potassium was lowered to 74 to 78 per cent (three rats) of the normal level 30 minutes after injection.

Since the fall and return to normal of serum potassium seemed to parallel the appearance and abatement of signs of prostigmine intoxication in dogs, it was thought that these signs might be altered by preventing the fall in potassium. Accordingly a fasting dog was given 500 mgm. potassium chloride

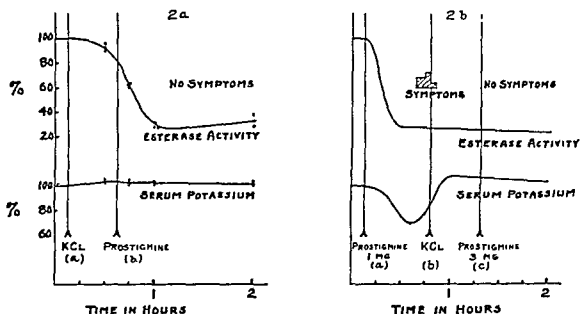


FIG. 2. PROSTIGMINE-POTASSIUM CHLORIDE ANTAGONISM

In 2a, potassium chloride (500 mgm.) was given at (a) by stomach tube, followed by prostigmine at (b). Note that there

symptoms. At (c), with subsequent fall of symptoms. At (b) 100 mgm. appearance of salivation, nasal discharge at (c), with no subsequent

by stomach tube, followed in 30 minutes by 1 mgm. prostigmine subcutaneously. The potassium level in the serum remained normal or was slightly elevated (fig. 2). Except for vomiting and defecation, there were none of the usual signs of prostigmine activity (i.e., salivation, lacrimation, nasal discharge or muscular tremors). The esterase was inhibited after prostigmine as usual. This experiment was repeated on three dogs with the same results. To determine whether potassium chloride would inhibit the signs of prostigmine after they had appeared 1 mgm. of prostigmine was administered and when the salivation, muscular tremors, etc. were at their maximum, 100 mgm. potassium chloride were injected intravenously, with disappearance of the



salivation, etc. in 1 to 3 minutes. Again, the gastro-intestinal tract activity persisted. This effect was demonstrated on 4 dogs. To one of these animals, after potassium chloride had stopped the usual reaction due to the initial dose of prostigmine, an additional 3 mgm. of the drug were injected; none of the usual responses to prostigmine were observed. These animals were not intolerant to prostigmine, since they exhibited perfectly typical responses to 1 mgm. dosages the day following the experiments combining potassium chloride and prostigmine.

TABLE 1

*Effect of prostigmine and guanidine on muscle potassium of rats*

WET WEIGHT		DRY WEIGHT			
Before drug	After drug	Before drug	Per cent H <sub>2</sub> O	After drug	Per cent H <sub>2</sub> O
Prostigmine					
0.33	0.50	1.20	75.8	1.93	75.5
0.33	0.57	1.29	74.0	1.57	74.0
0.30	0.33	1.24	75.2	1.43	75.6
0.31	0.47	1.24	75.5	1.36	76.0
0.27	0.30	1.27	75.6	1.94	75.2
0.31	0.37	1.26	76.0	1.68	76.2
0.28	0.35	1.27	75.6	2.03	75.0
Average....0.30	0.41	1.25		1.70	
Guanidine					
0.34	0.37	1.15	75.6	1.59	75.6
0.32	0.35	1.30	75.0	1.52	75.0
0.30	0.36	1.15	75.7	1.53	75.7
0.32	0.36	1.20	76.0	1.42	77.0
0.28	0.35	1.22	77.0	1.49	76.0
Average....0.31	0.36	1.21		1.51	

Potassium values are in grams K per 100 grams muscle, wet or dry weight.

2. *Effect of prostigmine and guanidine on muscle potassium.* Rats were used because of their size and ease of handling. In a control series of 5 rats no drug was injected and the muscles were removed under ether anesthesia. Potassium analyses of muscles from these rats showed no significant variations. The hamstring muscles of one leg were removed under ether anesthesia, the animal allowed to recover, and the drug administered in the same dosages as above. After 15 to 30 minutes the muscles of the second leg were removed under the same conditions. The samples were dissected free of connective tissue, the muscles were divided, one part dried to constant weight and the other analyzed directly for the wet-weight determination.

Both guanidine and prostigmine (table 1) increased muscle potassium. Dry



and wet figures are presented, the former showing the effect more clearly. Water content of the muscles was not significantly altered by administration of either drug.

3. *Effect of prostigmine on patients with Myasthenia gravis.* We have had access to only three such patients, and from none of these was it possible to obtain muscle biopsies. However, we were able to follow serum potassium and acetylcholine esterase before and after prostigmine. The results are

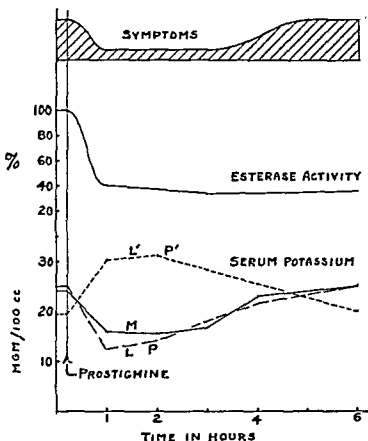


FIG. 3. EFFECT OF PROSTIGMINE ON SERUM POTASSIUM AND ESTERASE ACTIVITY OF PATIENTS WITH MYASTHENIA GRAVIS

These are typical curves showing the types of serum potassium reaction to prostigmine. All patient were done in the m

M—Patient M. uniformly showed a decrease. This patient had no therapy other than prostigmine. Patient L, whom we were able to follow for two months, had a fall in serum potassium after prostigmine until potassium chloride was

shown in figure 3. It should be observed that the relief of symptoms is much shorter than the duration of inhibition of the esterase, but does parallel quite closely the change in serum potassium. In patients with Myasthenia gravis the response of serum potassium to prostigmine was not constant. Patient M. uniformly showed a decrease. This patient had no therapy other than prostigmine. Patient L, whom we were able to follow for two months, had a fall in serum potassium after prostigmine until potassium chloride was



led to her medications. Potassium determinations on days when no potassium chloride was administered usually resulted in an elevated serum potassium curve after prostigmine. Patient P. had been on a combination prostigmine-potassium chloride regimen, and on re-admission to the hospital at first the elevated type of serum potassium response to prostigmine. However, after a week with no potassium chloride medication, there were occasions when the serum potassium decreased after prostigmine. The level of the initial serum potassium seemed to be an indication as to the response to prostigmine since there were usually elevated initial serum potassiums (25-30 mgm. per cent) when there was a fall after prostigmine. When initial readings were well within the normal range, an elevation in serum potassium occurred after the drug.

**DISCUSSION.** The important action of prostigmine may be the inhibition of the acetylcholine esterase, but this is by no means its only effect. The esterase activity of serum (Poncher and Wade (8)) and muscle (Jones and Smith (9)) from patients with Myasthenia gravis is not elevated. Goodman, Johnson and Gilman (10) reported a close relationship between serum esterase inhibition and relief of myasthenic symptoms. We were unable to confirm this, and examination of their charts reveals that symptoms had returned in some patients an hour or more before the esterase had shown any increase. Prostigmine, in doses producing comparable depression of esterase activity, was as effective as prostigmine (11). Additional evidence that prostigmine has actions other than its esterase inhibition may be inferred from its antagonism to curare. Recently Harris and Harris (12) have shown that curare inhibits the choline esterase. Guanidine and ephedrine, which are valuable remedies in Myasthenia gravis, have no effect on esterase activity. All of the drugs of benefit in Myasthenia gravis have some effect on isolated muscle. Harvey (3) found that guanidine sensitized the isolated frog muscle to potassium. Mendez and Ravin (13) reported muscle effects with prostigmine which they were unable to attribute to esterase inhibition. Ephedrine has long been known to stimulate isolated muscle (Kreitman (14)). Another action these drugs have in common is a lowering of the serum potassium and an increase in muscle potassium. This action has been reported with ephedrine and we have shown that guanidine and prostigmine produce the same effect in normal animals.

Cummins (15) has suggested that some defect in potassium metabolism exists in Myasthenia gravis. He found initially elevated muscle potassium, a decrease in potassium content after prostigmine. Accompanying this change in the muscle was an increase in serum potassium. We did not find this latter to be uniformly the case. In the three cases reported by Cummins, the initial serum potassiums were well within the normal range, and his patients had been treated with potassium chloride. Our patients showed the same response when these two conditions were met. At any rate, during



the change in serum potassium, whether elevated or lowered, the myasthenic patient experiences relief from his condition.

We are unable at the present time to do more than postulate that some abnormality in potassium metabolism is present in Myasthenia gravis. The drugs of use in this condition produce alterations in serum and muscle potassium of normal animals. Whether muscle concentration of potassium has much to do with the fatigue so characteristic of this condition may be questioned, since Miller and Darrow (16) demonstrated that wide variations of initial muscle potassium did not change the amount of swimming which rats were able to do before tiring.

We have no explanation to offer for the potassium chloride inhibition of the signs of prostigmine intoxication in dogs. It may be that maintenance of high serum potassium, which would prevent such a concentration gradient as would otherwise exist between serum and other phases, plays some rôle in alleviation of the signs.

#### SUMMARY

1. Prostigmine and guanidine produce a decrease in serum potassium in dogs and rats and an elevation of muscle potassium in rats.

2. The signs of prostigmine intoxication parallel more closely in dogs the depression of serum potassium than they do the acetylcholine esterase inhibition.

3. The serum potassium response to prostigmine in patients with Myasthenia gravis is not constant. The direction of change seems to depend on the initial level of serum potassium, and whether the therapy has included potassium chloride.

4. The duration of relief of symptoms in Myasthenia gravis after prostigmine follows more closely the change in serum potassium (whether elevated or lowered) than it does the decrease in activity of acetylcholine esterase.

We wish to express our appreciation to Dr. A. L. Sahs of the State University of Iowa Hospital for permitting us access to the neurological wards, to Dr. George Tice of the Pennsylvania Hospital, Philadelphia for sending us serum, and to the Hoffman-La Roche Company for its courtesy in supplying us with prostigmine.

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# THE EFFECTS OF MECHOLYL, POTASSIUM CHLORIDE AND PROSTIGMINE ON NEUROMUSCULAR ATROPHY AND REGENERATION<sup>1</sup>

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Received for publication September 2, 1941

The numerous publications concerned with the neurohumoral theory of excitation in peripheral structures have stimulated investigations in many allied fields of muscle and nerve physiology. A number of reports have appeared concerning the rôle of neurohumoral agencies and various peripherally acting drugs in altering the extent and velocity of muscular atrophy and the regenerative processes concomitant with reinnervation. According to Neuburger and Scholl (1) the administration of acetylcholine prevents the development of ankylosis and muscular atrophy which ordinarily results from immobilization of the hind limbs of rabbits. Levine, Hechter and Soskin (2) stated that the administration of prostigmine increased the fibrillary contractions and rate of atrophy and that atropine decreased the fibrillary contractions and rate of atrophy of the denervated gastrocnemius muscle of the rat. Wolf (3) reported that the oral administration of potassium chloride, acetyl-beta-methylcholine (mecholy) and prostigmine to cats and rats greatly shortened the duration of and hastened the recovery from lower motor neurone paralysis. Because of the possibility that some of these substances might prove to be valuable therapeutic measures in clinical cases of paralysis, it was deemed advisable to investigate further the action of chemical agencies upon the course of muscular atrophy and regeneration.

**METHODS.** In this investigation we have determined the effects of the combined administration of potassium chloride, mecholy and prostigmine upon the atrophy and regenerative changes associated with reinnervation of the denervated gastrocnemius muscle of the rat. To avoid the great variability in rates of regeneration encountered when the denervation of the muscle is accomplished by nerve section alone or section and resuture, we employed the method of nerve crushing. This was accomplished by crushing the exposed tibial nerve against a flat brass rod with the aid of a strong linen ligature. The nerve was released from its tie by cutting through the ligature down to the rod. Lesions were always made at the junction of the tibial and peroneal branches of the sciatic nerve in order to standardize the length of the nerve trunk involved. This technique gave complete motor axone separation and permitted a remarkably constant extent and rate of reinnervation. A number of criteria were employed to establish the

<sup>1</sup> Aided by a grant from The National Foundation for Infantile Paralysis, Inc.



completeness of the motor denervation. Electrical stimulation of the nerve above the lesion at the time of its preparation caused no visible contraction of the muscle. After allowing a period of three days for degeneration, stimulation of the nerve trunk below the lesion failed to elicit contraction in the muscle. The amount of atrophy and the length of the affected muscles as revealed by tests made at times prior to reinnervation were essentially equal to those found in the control muscle of the opposite limb which had been subjected to nerve section for the same period of time. Furthermore, both types of lesions allowed essentially the same decrease in creatine concentration of the affected muscles.

Measurements of muscle strength were made at 12, 18, 21 and 35 days after establishment of the lesions. The animals were placed under light ether anaesthesia and the gastrocnemius muscles and tibial nerves of the operated and control limbs were exposed. The tendon of Achilles was cut and attached to a Blix type torsion rod. A portion of the mur was exposed and fixed in a rigid clamp, care being taken to avoid interference with the blood supply to the muscle. The muscle was directly stimulated through two needle electrodes which pierced it, one at the tendon and the other at the origin. Adjustable silver electrodes were placed in contact with the tibial nerve. A short bout of superaximal stimuli, either from an inductorium or as condenser discharges, was delivered to the muscle and nerve. The frequency and strength were such as were found to be adequate to give a maximal tetanus tension. The extent of muscle shortening was measured from optical records. Muscle strength was taken to be the maximal tension developed in response to stimuli applied either directly to the muscle or to its motor nerve. After these measurements were made the animal was killed by bleeding, the gastrocnemius muscles were carefully dissected, weighed and analyzed for creatine. These procedures were found to yield quite consistent values for the strength of normal muscle, and the values obtained by activation through the motor nerve approached those elicited by direct muscle stimulation.

Standard lesions were made in the tibial nerves of twenty-eight animals which received 47 mgm. of potassium chloride, 4.8 mgm. of mechoyl and 0.72 mgm. of prostigmine per day in their drinking water. Twelve of the animals were housed in individual cages and the drugs were dissolved in slightly less than the volume of water that was consumed by the animals in preliminary twenty-four hour periods. When the drinking tubes were ready of the solution they were filled with water for the rest of the twenty-four hour period. The remainder of the experimental animals were divided into two groups and allowed to share common water bottles. The average amounts of the drugs consumed by rats housed in group cages were the same as those ingested by animals in individual cages. A total of forty-six animals were subjected to crushing of the tibial nerves and served as untreated controls. Care was taken to select experimental and control animals from the same age group.

**RESULTS AND DISCUSSION.** The results as a whole are consistent in showing the absence of any effects from the administration of mechoyl, potassium chloride and prostigmine upon the rate of muscular atrophy and recovery from lower motor neurone lesions. No differences were to be found between the treated and untreated animals as to the time at which the earliest signs of functional reinnervation appeared. This was measured by determining the earliest time at which stimulation of the motor nerve would cause the slightest detectable movement of the exposed gastrocnemius muscle. This test of initial reinnervation was negative until twelve to fourteen days after



establishment of the lesion in both the control and experimental animals. The amount of tension developed by the muscles in response to stimulation of their regenerating motor nerves increased rapidly from day to day and could be satisfactorily measured as early as eighteen days after the lesions had been made. Average values together with the standard errors for the studies that were made on the paralyzed and control gastrocnemii from treated and untreated animals are summarized in table 1, in which the weight of the muscle with a lesion in the motor nerve is compared with that of its unoperated contralateral control. It is to be noted that the denervated muscles continued

TABLE 1

*A summary of the average values for muscles of treated and control animals*

CONDITION OF ANIMALS	TIME AFTER LESION	PER CENT WEIGHT LOSS	TENSION PER GRAM MUSCLE WHEN ACTIVATED THROUGH				RELATIVE STRENGTH OF DENERVATED MUSCLE WHEN ACTIVATED THROUGH		CREATINE*	
			Nerve		Muscle		Nerve	Muscle	Crushed	Intact
			Crushed	Intact	Crushed	Intact				
	days									
Treated	12	31.7			1042	1760		40.3	340	439
		±1.4			±66	±56		±2.2	±13	±4
Control	12	29.9			1174	1773		47.4	336	453
		±3.1			±62	±56		±2.4	±1	±5
Treated	18	35.5	585	1575	1300	1810	23.5	47.7	355	473
		±2.6	±54	±94	±44	±75	±2.8	±2.5	±6	±5
Control	18	36.3	570	1623	1379	1867	22.3	47.6	361	462
		±1.2	±72	±42	±72	±66	±2.4	±2.2	±10	±12
Treated	21	38.4	622	1667	1221	1864	23.9	40.6	353	473
		±1.3	±51	±29	±45	±40	±2.3	±1.7	±7	±4
Control	21	36.5	613	1630	1288	1827	24.3	45.0	370	461
		±1.9	±80	±14	±40	±47	±3.3	±1.9	±7	±3
Treated	35	20.9	1193	1511	1482	1863	61.8	62.2	426	476
		±4.6	±153	±89	±188	±132	±3.5	±4.1	±5	±8
Control	35	20.0	1291	1418	1579	1725	58.1	63.9	416	456
		±3.7	±3	±178	±4	±241	±4.0	±2.6	±6	±4

\* Mgm. per 100 grams muscle.

† Expressed as per cent of that found in contralateral control.

to lose weight and strength for some time after positive signs of partial re-innervation were present. This is believed to be due to the fact that some axones made anatomical and functional contact with muscle fibers much sooner than others because of the necessity of certain fibers regenerating over a greater distance than others. It would appear as if for a time certain fibers were undergoing further atrophy while others, having established neural contacts, were exhibiting regeneration. A survey of the muscle weight values made at various times after denervation indicates that essentially the same rate of atrophy and subsequent regeneration had occurred in the treated and



reated control animals. No differences were noted in the amount of reduction in the creatine concentration of muscles from control and experimental subjects. The tension studies indicate that the strength of the muscles in treated animals did not differ from that found in the untreated controls. This was true for the responses elicited through nerve stimulation as well as those brought about by direct muscle stimulation. It must be concluded from these aspects of the criteria employed that the administration of mecholyl, potassium chloride and prostigmine failed to arrest the muscular atrophy occurring after denervation and, likewise, was ineffective in hastening recovery from paralysis.

One can only speculate as to reasons for the failure to confirm the reports of other investigators concerning beneficial effects of these substances in delaying muscular atrophy and hastening recovery from paralysis. Undernutrition and fasting cause unequal amounts of material to be lost from normally innervated and denervated muscle according to Hines and Knowlton (4). Errors in estimating the rates of atrophy and regeneration may therefore be made whenever the administration of drugs and other experimental conditions result in a significant change in body weight during the period of an experiment. In our studies the body weights of the animals remained essentially the same throughout the experimental periods.

Wolf (3) preferred alcohol injections to nerve section and resuture as a means of producing lower motor lesions because the latter procedure allowed possible complications due to shifting and twisting of the severed parts. However, he admits that there are probably variations in the number of fibers caught in the degenerative process induced by the injection technique. It is believed that the method of producing nerve lesions employed in this investigation gave complete degeneration and also allowed for a better preservation of alignment than the cutting and resuture procedure.

Wolf (3) postulated an additional factor responsible for the variations noted in different animals. He suggested that all of the medicated animals may not have received the same dosage and that those that consumed the larger amounts might have experienced a depressing effect of the cholinergic facilitators. In this investigation no significant differences were noted between the experiments in which the animals received a known dosage and those on groups in which the individual dosage might have varied.

Other investigators have employed somewhat different criteria in evaluating the recovery from paralysis concomitant with reinnervation. In some instances they have been subjective in character and have not included quantitative measurements of muscle weight and strength. Our experiments do not exclude the possibility that treatment with such agencies may enhance the actual usage of muscle through reflex arcs newly established through reinnervation. However, if such facilitation occurred, it did not reflect itself in any demonstrable change in the composition, strength or weight of the muscles involved.



## SUMMARY

The daily oral administration of 4.8 mgm. of mecholyl, 47 mgm. of potassium chloride and 0.72 mgm. of prostigmine to adult albino rats with lesions in their tibial nerves neither delayed muscular atrophy nor hastened neuromuscular regeneration. The criteria employed in the studies include quantitative determinations of the weight changes, creatine concentration and strength of the gastrocnemius muscle at various times after denervation. A discussion is included of the reports of other investigators concerning the favorable influence of these drugs on muscular atrophy and recovery from paralysis.

We are indebted to Hoffmann-La Roche, Inc. for their generous supply of prostigmine used in this investigation.

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# SULFAPYRAZINE (2-SULFANILAMIDOPYRAZINE): ITS ANTI-PNEUMOCOCCAL ACTIVITY AS COMPARED WITH THAT OF SULFAPYRIDINE, SULFATHIAZOLE AND SULFADIAZINE

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Received for publication September 19, 1941

Sulfapyrazine (2-sulfanilamidopyrazine), an isomer of sulfadiazine, was synthesized recently by Ellingson (1). Preliminary clinical and experimental studies (2, 3) have indicated that this new compound has considerable anti-pneumococcal activity. How this activity compares with that of other sulfonamides of established therapeutic value, such as sulfapyridine, sulfathiazole and sulfadiazine, has not yet been determined. The present report concerns the results of such a comparison.

**EXPERIMENTAL.** *A. The antipneumococcal activity of sulfapyrazine, sulfadiazine, sulfathiazole and sulfapyridine in vivo.*

*In vivo* experiments were carried out with each of three different strains of pneumococcus. These strains, one each of types I, II and III, were at maximum virulence and invasiveness when used, having undergone more than 400 passages through normal mice. These particular organisms have been used in chemotherapeutic studies in this laboratory for nearly two years; consequently, their responses to different sulfonamides, both *in vivo* and *in vitro*, have been determined with reasonable certainty. *In vivo*, the type I strain has been found to be moderately sensitive, whereas the type II and III organisms have been moderately resistant (4).

In each *in vivo* experiment, 260 white mice (males, weighing 18 to 22 grams) were infected intraperitoneally, each mouse receiving  $10^{-6}$  cc. of a 12- to 14-hour blood broth culture of the desired organism. Twenty of these mice were retained as untreated controls. The remaining 240 animals were divided into 4 equal groups. Each group was treated with a different drug,<sup>1</sup> 30 mice receiving 5 mg. doses and 30 receiving 10 mg. doses. These quantities, suspended in 10 per cent acacia, were administered by stomach tube 2, 8, 14, and 22 hours after infection and every eight hours thereafter for a maximum of five additional days, or as long as the mice survived.

The results of these experiments, recorded in table 1, show that sulfapyrazine was as effective as sulfadiazine against experimental pneumococcal infections, both in curative and in life-prolonging action. These two drugs were con-

<sup>1</sup> We are indebted to Merck and Company, Rahway, N. Y., for the sulfathiazole used in these experiments; to the American Cyanamid Corporation, Stamford, Conn., for the sulfadiazine; and to Mead Johnson and Company, Evansville, Indiana, for the sulfapyrazine.



siderably more effective than either sulfapyridine or sulfathiazole, particularly when used in 5 mgm. doses.

TABLE 1

*The effectiveness of sulfapyrazine, sulfadiazine, sulfapyridine and sulfathiazole against experimental infections with Types I, II and III pneumococci*

NUMBER OF MICE INFECTED	TREATMENT*		NUMBER OF DEATHS DAYS AFTER INFECTION								HOURS SUR- VIVAL OF MICE THAT DIED	SURVIVORS	
	Drug†	Dose	1	2	3	4	5	6	7-10	11-20		Num- ber	Per cent
Type I, McGovern: 550 organisms in infecting dose													
30	SPZ	5	0	0	0	0	0	0	2	2	240	26	87
30	SD	5	0	0	0	1	0	1	2	2	184	24	80
30	SP	5	0	0	1	3	1	0	13	1	159	11	37
30	ST	5	0	0	1	1	0	1	14	2	177	11	37
30	SPZ	10	0	0	0	0	0	0	1	2	233	27	90
30	SD	10	0	0	0	0	0	1	3	0	188	26	87
30	SP	10	0	0	0	0	0	0	6	0	179	24	80
30	ST	10	0	0	0	0	0	1	11	0	178	18	60
20	Controls	0	0	20	0	0	0	0	0	0	30	0	0
Type II, CH: 150 organisms in infecting dose													
30	SPZ	5	0	0	0	0	1	0	26	0	164	3	10
30	SD	5	0	0	0	0	0	3	26	0	164	1	3
30	SP	5	0	0	6	3	6	8	7	0	114	0	0
30	ST	5	0	7	13	3	5	2	0	0	72	0	0
30	SPZ	10	0	0	0	0	1	2	25	0	168	2	7
30	SD	10	0	0	0	0	1	3	23	0	170	3	10
30	SP	10	0	0	0	0	0	9	18	0	149	3	10
30	ST	10	0	0	4	3	3	9	11	0	127	0	0
20	Controls	0	3	17	0	0	0	0	0	0	28	0	0
Type III, CHA: 340 organisms in infecting dose													
29	SPZ	5	0	0	0	0	0	0	27	1	189	1	3
30	SD	5	0	0	1	1	0	1	27	0	175	0	0
30	SP	5	0	1	0	2	0	2	24	0	154	1	3
30	ST	5	0	3	12	6	7	2	0	0	80	0	0
29	SPZ	10	0	0	0	0	0	0	27	1	198	1	3
29	SD	10	0	0	0	1	0	1	24	3	193	0	0
30	SP	10	0	0	0	0	1	0	28	0	166	1	3
30	ST	10	0	0	0	3	9	7	11	0	129	0	0
20	Controls	0	15	5	0	0	0	0	0	0	23	0	0

\* Dose of drug administered at 2, 8, 14 and 22 hours after infection and every 8 hours thereafter for five days or as long as the mice survived.

† SPZ = Sulfapyrazine; SD = Sulfadiazine; SP = Sulfapyridine; ST = Sulfathiazole.

Against infections with type I strain McGovern, the most sensitive of the three organisms studied, 5 mgm. doses of sulfapyrazine and sulfadiazine cured



87 and 80 per cent of the mice, respectively, whereas similar doses of either sulfapyridine or sulfathiazole cured only 37 per cent of the animals. None of the drugs cured a significant number of mice infected with type II strain CH and type III strain CHA, the moderately resistant organisms. However, sulfapyrazine and sulfadiazine prolonged life more than did sulfapyridine and sulfathiazole. Thus in the experiment with the type II strain, mice treated with 5 mg. doses of either sulfapyrazine or sulfadiazine lived on the average 164 hours, whereas mice treated with similar amounts of sulfapyridine and sulfathiazole lived only 114 and 72 hours, respectively. Similar observations were made in the experiment with the type III strain.

It is interesting to note that in each of the experiments 5 mgm. doses of sulfapyrazine were about as effective as 10 mgm. doses. This finding was characteristic of sulfadiazine also, but was in marked contrast to the results with sulfapyridine and sulfathiazole, these latter drugs being considerably more effective in 10 mgm. doses than in those of 5 mgm. At least a partial explanation for these observations will be apparent later.

*B. The antipneumococcal activity of sulfapyrazine, sulfadiazine, sulfathiazole and sulfapyridine in vitro.* The growth-inhibiting properties of the four drugs were compared *in vitro*, the same strains of pneumococcus being used as in the *in vivo* experiments. Previous studies (4) with sulfapyridine have shown that the *in vitro* sensitivities of these organisms are different from their *in vivo* sensitivities. *In vitro*, the type II strain is highly sensitive to the sulfonamides and the type I and III strains are only moderately sensitive. (*In vivo*, as stated above, the type I strain is highly sensitive and the type II and III organisms moderately resistant.)

In each *in vitro* experiment, 9 cc. quantities of beef heart infusion broth (4), containing the drugs in varying concentrations, were placed in test tubes, enriched with 0.2 cc. of rabbit blood and inoculated with 1 cc. of a  $10^{-5}$  beef heart broth dilution of a 12- to 14-hour blood broth culture of the desired organism. Observations on growth were made after twelve and twenty-four hours incubation at 37.5°C. Two criteria were used as an index of growth: (1) turbidity, and (2) change in the color of hemoglobin from bright red to chocolate brown. These criteria were adopted after numerous growth curve studies, in which the above medium has been used, had shown that when the population of the culture exceeded 5,000,000 pneumococci per cubic centimeter, the hemoglobin-free supernatant liquid became turbid and the hemoglobin itself changed color from bright red to chocolate brown. Although crude, these criteria suffice for the present purposes since they show that the population of the culture has increased at least ten thousand-fold. Experiments with each strain were repeated several times.

The results of a typical experiment, summarized in table 2, show that against 2 of the strains sulfapyrazine was as effective as sulfathiazole and was more effective than either sulfapyridine or sulfadiazine, particularly the latter.



against the third organism, sulfapyrazine was slightly less effective than sulfathiazole, but was equal to sulfapyridine and more effective than sulfadiazine.

Thus at the end of 24 hours incubation, growth of the type II strain (the most sensitive strain *in vitro*) was checked by 1.25 mgm. per cent sulfapyrazine and sulfathiazole, 2.5 mgm. per cent sulfapyridine and 5 mgm. per cent sulfapyrazine. At the same time, growth of the type I strain—a less sensitive organism—was inhibited by 2.5 mgm. per cent sulfapyrazine and sulfathiazole,

TABLE 2

*The effect of sulfathiazole, sulfapyrazine, sulfapyridine and sulfadiazine on the in vitro growth of Types I, II and III pneumococci*

ORGANISM	INOCULUM: NUMBER OF PNEUMOCOCCI PER CC. CULTURE	DRUG*	GROWTH									
			After 12 hours incubation					After 24 hours incubation				
			Conc. Drug (mgm. per cent)					Conc. Drug (mgm. per cent)				
			0	1 25	2 5	5 0	10 0	1 25	2 5	5 0	10 0	
I, McGovern	650	Control	+	—	—	—	—	+	—	—	—	
		ST		+	—	—	—	+	—	—	—	
		SPZ		+	—	—	—	+	+	—	—	
		SP		+	—	—	—	+	+	—	—	
		SD		+	+	—	—	+	+	+	—	
II, CH	220	Control	+	—	—	—	—	—	—	—	—	
		ST		—	—	—	—	—	—	—	—	
		SPZ		—	—	—	—	—	—	—	—	
		SP		—	—	—	—	+	—	—	—	
		SD		+	—	—	—	+	+	—	—	
III, CHA	320	Control	+	—	—	—	—	+	—	—	—	
		ST		—	—	—	—	+	+	—	—	
		SPZ		—	—	—	—	+	+	—	—	
		SP		—	—	—	—	+	+	—	—	
		SD		+	+	—	—	+	+	+	—	

\* ST = Sulfathiazole; SPZ = Sulfapyrazine; SP = Sulfapyridine; SD = Sulfadiazine.

mgm. per cent sulfapyridine and 10 mgm. per cent sulfadiazine. Similarly, growth of the type III pneumococcus was inhibited by 2.5 mgm. per cent sulfathiazole, 5 mgm. per cent sulfapyrazine and sulfapyridine and 10 mgm. per cent sulfadiazine.

C. Concentrations of sulfapyrazine, sulfadiazine, sulfathiazole and sulfapyridine maintained in the blood. The concentrations of the four sulfonamides, maintained in the blood of treated mice, were determined in order to evaluate the results of the therapeutic experiments properly. An attempt was made to ascertain the maximum and minimum concentrations of these



drugs during the first two days of therapy. Groups of normal mice were treated with 5 and 10 mgm. doses of the respective drugs, according to the treatment schedule used during the first 48 hours of the *in vivo* experiment. At two and eight hours after the fourth and seventh doses, groups of 5 mice receiving a given treatment were sacrificed, heart blood samples were collected and pooled, and analyses for free drug were carried out according to the method of Bratton and Marshall (5).

The results of these analyses are recorded in table 3. The most important findings can be summarized as follows: (1) Sulfapyrazine concentrations were more nearly constant than those of any other drug, levels eight hours after treatment being nearly identical with those at two hours. Five milligram

TABLE 3

*Concentrations of sulfapyrazine, sulfadiazine, sulfathiazole and sulfapyridine in the blood of treated mice*

DRUG	DOSAGE*	CONCENTRATION OF SULFONAMIDE (MG. PER CENT)†			
		Hours after fourth dose		Hours after seventh dose	
		2	8	2	8
Sulfapyrazine	mgm. 5	6.6	6.0	8.3	7.0
	10	8.0	7.5	9.6	10.0
Sulfadiazine	5	18.0	10.7	24.0	15.2
	10	23.2	13.2	29.0	19.2
Sulfathiazole	5	4.9	trace	3.3	trace
	10	13.3	2.3	5.5	2.8
Sulfapyridine	5	9.2	3.2	8.9	3.3
	10	19.8	6.5	19.0	8.7

\* The drugs were administered at 0, 6, 12, and 20 hours for the first four doses and at 28, 36, and 44 hours for the remaining three doses.

† Concentration of free drug found in pooled heart blood from five treated mice.

doses of sulfapyrazine produced blood levels similar to 10 mgm. doses; this explains why these doses had similar therapeutic effects. (2) Sulfadiazine concentrations were considerably higher than those of the other drugs, both at two and eight hours after treatment; this may account for the finding that sulfadiazine was more effective *in vivo* than its growth-inhibiting activity *in vitro* would indicate. Five and 10 mgm. doses of sulfadiazine produced similar blood levels. (3) Sulfapyridine concentrations varied considerably at two- and eight-hour periods; two hours after 10 mgm. doses, sulfapyridine levels were much higher than sulfapyrazine, while at eight hours they were similar; eight hours after 5 mgm. dosage, sulfapyridine concentrations were only half the sulfapyrazine levels. (4) Sulfathiazole concentrations fluctuated more than those of the other drugs; eight hours after 5 mgm. doses, only



traces of sulfathiazole were present in the blood; eight hours after 10 mgm. doses, sulfathiazole concentrations were approximately one third those of sulfapyrazine and sulfapyridine.

COMMENT. The data presented above show that sulfapyrazine compares favorably with sulfadiazine, sulfathiazole and sulfapyridine in the treatment of experimental pneumococcal infections. Doubtless some of the effectiveness of sulfapyrazine can be attributed to its capacity to inhibit growth of pneumococci—a capacity which is second only to that of sulfathiazole. Another factor, possibly of greater importance, is sulfapyrazine's property of being retained in the blood at uniformly high and effective levels throughout the treatment period. Undoubtedly this property is largely responsible for the finding that dose for dose sulfapyrazine is more effective than sulfapyridine and sulfathiazole, particularly the latter.

Whether sulfapyrazine will be a useful addition to the sulfonamide group would seem to depend largely upon its toxicity and the regularity with which it is absorbed. These subjects are being studied thoroughly by Ruegsegger and coworkers. Preliminary experiments (2) have indicated that sulfapyrazine is about as toxic for mice as sulfadiazine, and that it has a relatively low toxicity for rats, dogs and monkeys. A preliminary clinical study (2) has indicated that human patients tolerate sulfapyrazine about as well as sulfadiazine and better than sulfapyridine and sulfathiazole. If absorption and excretion of sulfapyrazine in humans lead to the uniform blood levels found in the mouse, this drug may be more useful clinically than sulfadiazine since sulfapyrazine has the greater capacity to inhibit growth of pneumococcus.

#### SUMMARY

The antipneumococcal activity of sulfapyrazine was compared with that of sulfadiazine, sulfathiazole and sulfapyridine. Sulfapyrazine was as effective as sulfadiazine against experimental pneumococcal infections and was somewhat more effective than sulfapyridine and sulfathiazole. Sulfapyrazine was only slightly less effective than sulfathiazole in inhibiting the growth of pneumococcus *in vitro*, and was more effective than either sulfapyridine or sulfadiazine. Sulfapyrazine concentrations in the blood of treated mice were maintained at a uniform level throughout the treatment period. This latter finding, together with the marked growth-inhibiting properties of this drug, probably accounts for its effectiveness against experimental infections.

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